University of Alberta

The role of RasGRP1 and RasGRP3 in B cells

by

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This thesis is dedicated to Heidi and Emma:
For if I had been with them, as I desired and they deserved,
this thesis would not have been studied or written.

Abstract

The RasGRPs are a family of Ras activators that possess diacylglycerol-binding C1 domains in addition to calcium-binding EF hands. In T cells, RasGRP1 links T cell receptor signaling to Ras. T cell receptor and B cell receptor (BCR) signaling share a great number of similarities. Therefore, I set out to investigate the possible role of RasGRPs in linking BCR signaling to Ras. Here I show that murine B cells express both RasGRP1 and RasGRP3. Using *Rasgrp1*, *Rasgrp3* single and double null mutant mice, I analyzed the role of these proteins in signaling to Ras in B cells. RasGRP1 and RasGRP3 both contribute to BCR-induced Ras activation, although RasGRP3 alone is responsible for basal Ras activation in unstimulated cells. RasGRP-mediated Ras activation is not essential for B cell development, as this process occurs normally in double mutant mice. Additionally, the absence of RasGRP1 expression did not adversely affect humoral immune responses, although the absence of RasGRP3 expression resulted in isotype specific defects in antibody production.

Remarkably, while aged $Rasgrp1^{-/-}$ mice develop late-onset autoimmunity characterized by the presence of anti-nuclear antibodies, the additional loss of RasGRP3 expression inhibited this phenotype. $Rasgrp1^{-/-}$ $CD4^{+}$ T cells induced greater B cell proliferation than wildtype T cells. This greater B cell induction is mediated in part by the increased capacity of these cells to produce IL-4. B cell proliferation induced by BCR ligation plus IL-4 was found to be RasGRP1- and RasGRP3-dependent, while anti-CD40 plus IL-4 and LPS-induced proliferation does not require the expression of these proteins. BCR stimulation plus IL-4 results in sustained cyclin D2 production in

wildtype B cells. However, there is no induction of this protein in double knockout B cells treated with these agents. This lack of induction likely explains the inability of these mitogens to induce the proliferation of these B cells. As such, I propose that the autoimmunity induced by the lack of RasGRP1 expression is suppressed in double knockout mice due to the diminished ability of B cells from these mice to upregulate cyclin D2 expression and proliferate upon IL-4 and BCR stimulation.

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List of abbreviations and symbols

7-AAD	7-Amino-actinomycin D
aPKC	Atypical PKC
BCR	B cell receptor
BrdU	Bromodeoxyuridine
C1	Conserved region 1 of the PKC
cdk	Cyclin-dependent kinase
CFSE	5- (and 6-) carboxyfluorescein diacetate succinimidyl ester
cPKC	Conventional PKC
DAG	Diacylglycerol
DGK	Diacylglycerol kinase
ER	Endoplasmic reticulum
Erk	Extracellular-regulated kinase
GAP	GTPase activating protein
γc	Common gamma chain
GEF	Guanyl nucleotide exchange factor
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IP3	Inositol 1,4,5-trisphosphate
IL-4	Interleukin-4
Mapk	Mitogen activated protein kinase
MBP	Maltose-binding protein
Mek	Mitogen activated protein kinase/extracellular signal-regulated kinase kinase
nPKC	Novel PKC
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase

PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PH	Pleckstrin homology
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
Ras	Rat sarcoma transforming sequence
RasGRP	Ras guanyl nucleotide releasing protein
RBD	Ras-binding domain
SH2	Src homology 2
SH3	Src homology 3
SMAC	Supramolecular activation cluster
Sos	Son of Sevenless
TCR	T cell receptor
TD	T cell-dependent
TI-I	T cell-independent type I
TI-II	T cell-independent type II

Chapter 1
Introduction

Chapter

1

Introduction

1.1 Overview

The immune system is composed of a wide variety of cell types that cooperate in order to clear pathogens from the body. An overactive immune system can result in autoimmunity, while reduced immune system function can lead to immune deficiency. The immune system is finely tuned, allowing for the appropriate strength of response to each pathogen. This response is triggered by receptors on the surface of immune cells that recognize the pathogen. Signals travel across the plasma membrane to a wide range of cellular proteins allowing for activation, proliferation and effector function of the immune cells.

CD4$^+$ T cells, often referred to as helper T cells (Th), are regulators of the immune system. They have surface receptors that allow them to recognize proteins encoded by pathogens (antigens), and following receptor ligation they upregulate the expression of surface molecules as well the production of cytokines which allow them to modulate the response of other immune cells. One cell type affected by the Th cell co-stimulatory signals is B cells, which also have antigen receptors that allow them to recognize pathogens. When activated, B cells secrete antibodies that bind to the pathogen inducing damage and allowing other cells to recognize the pathogen. A large body of research has focused on the signaling pathways initiated by the antigen receptors on both T cells (T cell receptor, TCR) and B cells (B cell receptor, BCR), and how these signals lead to effector functions.

In 1990, Downward and colleagues discovered that upon TCR stimulation a small G protein, Ras, is activated (Downward et al. 1990). This was the first identification of a biologically relevant stimulus that induced Ras activation. This discovery set in motion research aimed at understanding the regulation of Ras activation as well as the effects of this activation in immune cells as well as the other cells of the body.

1.2 Ras

The Ras genes were first discovered in retroviruses capable of inducing tumor formation in rats (Harvey 1964; Kirsten et al. 1966). These rat sarcoma causing genes were later found to be mutated forms of cellular genes (Langbeheim et al. 1980; Ellis et al. 1981), and these cellular genes were found to be mutated in a large number of human cancers (Bos 1989). This latter finding generated a great deal of interest in the study of these proteins. The Ras superfamily consists of over 150 small G proteins that bind to GTP and hydrolyze it to GDP (Figure 1-1). The Ras subfamily contains 13 members divided into 5 groups (Ehrhardt et al. 2002). The initially discovered Ras proteins are included in a group of this subfamily referred to as the classical Ras proteins. These include H-Ras, N-Ras, and the two alternatively spliced forms of K-Ras (K-Ras 4A and K-Ras 4B). Also included in the Ras subfamily are the Rap (Rap1A, Rap1B, Rap2A, and Rap2B), R-Ras (R-Ras, and TC21/R-Ras2), M-Ras/R-Ras3 and Ral (RalA and RalB) proteins. Throughout this dissertation, Ras is used to collectively refer to the classical Ras proteins, and more specifically N-Ras and K-Ras 4B as they are the only classical Ras proteins expressed in T cells and B cells (Leon et al. 1987; Downward et al. 1990; Pells et al. 1997; Wang et al. 2001; Plowman et al. 2003; Perez de Castro et al. 2004).

Membrane localization of the Ras proteins is key to their function. N-Ras and K-Ras 4B both contain carboxy-terminal CAAX motifs (cysteine followed by two aliphatic amino acids and one random amino acid), which are post-translationally modified resulting in prenylation and cleavage to release the AAX motif. In addition to these modifications, other motifs contribute to membrane association including palmitoylation of cysteines near the carboxy-terminus in N-Ras (as well as H-Ras and K-Ras 4A), and a polybasic region in K-Ras 4B (Hancock et al. 1989; Hancock et al. 1990). These motifs result in the differential trafficking of Ras proteins, with N-Ras traveling from the endoplasmic reticulum (ER) to the Golgi before proceeding to the plasma membrane, while K-Ras 4B bypasses the Golgi (Choy et al. 1999; Apolloni et al. 2000). These Ras proteins also display different localization within regions of the plasma membrane, with K-Ras 4B being excluded from lipid rafts (Roy et al. 1999; Prior et al. 2001; Ehrhardt et al. 2004), a key membrane domain in immune receptor signaling (Jacobson et al. 1999; Pierce 2002). There is also evidence of Ras signaling on endomembranes, further highlighting the importance of the differential trafficking and localization of Ras proteins (Chiu et al. 2002; Silvius 2002; Bivona et al. 2003; Caloca et al. 2003).

1.3 Ras effector pathways

While in the GTP-bound state, the globular structure of Ras is altered from that of the GDP-bound state such that it allows for effector binding (Herrmann 2003). Ras effectors are diverse in function, although most contain a variant of the Ras binding domain (RBD) that allows them to bind specifically to the GTP-bound form of Ras. Given the large number of Ras effectors identified, it is unlikely that Ras activation in every cell type results in the activation of every effector. Instead, the effectors activated are cell type specific and signal dependent. It is also possible that proteins identified as Ras effectors act downstream of other Ras family members, as they share the core Ras effector region. Included here is a selection of the best-characterized Ras effectors.

Raf

The Raf family is the best-characterized Ras effector family. A-Raf, B-Raf and Raf-1 (C-Raf) share a similar domain structure consisting of three conserved regions (CR1-3). CR1 contains a Ras binding domain (RBD) and a cysteine rich domain (CRD). While the CRD interacts with Ras independent of the specific guanyl nucleotide bound to Ras, the RBD binds Ras only when it is in the active, GTP-bound conformation (Fabian et al. 1994). Despite the similarities of the domain structures within the Raf family, Ras proteins have differing affinities for the three Rafs (Yan et al. 1998; Voice et al. 1999; Weber et al. 2000). These differing activities could help to explain why mice deficient in each classical Ras family member have different phenotypes (Koera et al. 1997; Esteban et al. 2001; de Castro et al. 2003), as do mice deficient in each of the three Raf proteins (Pritchard et al. 1996; Wojnowski et al. 1997; Wojnowski et al. 1998). Raf binding to membrane bound Ras-GTP enables Raf phosphorylation by other kinases. The pattern of Raf phosphorylation is complex, with both positive and negative regulatory sites existing in each of the family members (Chong et al. 2003; Wellbrock et al. 2004). Inhibitory phosphorylation prevents Ras-Raf interactions, while activating phosphorylation increases the catalytic activity of the kinase domain located in CR3. While phosphorylation of A- and Raf-1 are similar, the larger B-Raf has fewer activating phosphorylation sites, and one of its sites is constitutively phosphorylated (Mason et al. 1999). This decreased regulation of B-Raf correlates with an increased basal activity and could help to explain the prevalence of B-Raf mutations in human cancers (Davies et al. 2002).

Active Raf (a mitogen activated protein kinase kinase kinase, Mapkkk) phosphorylates two serine residues on Mek (a Mapkk), causing Mek to be activated and phosphorylate Erk (extracellular regulated kinase, a Mapk)(Dent et al. 1992; Howe et al. 1992; Kyriakis et al. 1992). Active Erk, dually phosphorylated on the TEY (threonine-glutamate-tyrosine) motif, is then capable of serine/threonine phosphorylation and activation of a number of effectors. These include proteins with enzymatic activity (p90Rsk, p70 S6 kinase (Sturgill et al. 1988), cytosolic phospholipase A_2 (Lin et al. 1993)), transcription factors (Elk-1 (Janknecht et al. 1993; Marais et al. 1993), SAP-1 (Janknecht et al. 1995), STATs (Decker et al. 2000)) as well as many others (Lewis et al.

2000). Erk-mediated Elk-1 activation causes the transcription of c-fos, a transcription factor that heterodimerizes with c-jun to form AP-1 (Whitmarsh et al. 1996). AP-1 , in collaboration with various other transcription factors, alters the transcription of a wide variety of genes including cytokines (IL-2, IL-3, IL-4, GM-CSF), receptors (FasL, Fas, CD44, CD69), and cell cycle proteins (cyclin D1, cyclin D2) among others (Rooney et al. 1995; Kerkhoff et al. 1998; Macian et al. 2000; Eferl et al. 2003). The result is a diverse set of effector functions and integration with other signaling pathways.

Mekk1

Mekk1 is a Mapkkk with broader substrate specificity than Raf. Mekk1 has been found to associate with and phosphorylate Mkk4, in addition to Mek1, which are the upstream kinases involved in the activation of the Mapks Jnk and Erk respectively (Lange-Carter et al. 1993; Minden et al. 1994; Yan et al. 1994; Xu et al. 1995; Karandikar et al. 2000). Mekk1 possesses a proline-rich region, an E3 ubiquitin ligase ring finger domain, a ubiquitin interaction motif, and a serine/threonine kinase domain (Xu et al. 1996; Lu et al. 2002). The ability of Mekk1 to ubiquitinate Erk, and thus target it for degradation, suggests that Mekk1 primarily activates the Jnk pathway *in vivo*. There is evidence that, in spite of Mek1 phosphorylation, Mekk1 does not cause significant Erk activation (Xu et al. 1995; Yujiri et al. 1998). Mekk1 has been shown to interact with Ras in a GTP-dependent fashion despite lacking any obvious RBD (Russell et al. 1995). Mekk1 can also bind to the GTP-bound forms of the small G proteins Cdc42 and Rac1 *in vitro* (Fanger et al. 1997). In addition, RhoA, another Ras superfamily member, has been shown to bind to Mekk1 and regulate its kinase activity (Gallagher et al. 2004). Thus, it is possible that Mekk1 could link Ras activation with Mkk4-Jnk. Jnk could then in turn cause the activation of several transcription factors including c-Jun, a component of the AP-1 complex (Davis 2000).

PI3K

Phosphatidylinositol (PI) is a key lipid involved in signaling downstream of many receptors. The phosphorylation state of this lipid regulates interactions with many proteins, and cleavage of the inositol ring can generate the second messengers DAG and calcium (see section 1.5.1). Phosphoinositide 3-kinase (PI3K) phosphorylates PIP2 at the 3' position on the inositol ring, allowing for recognition of this lipid by proteins with pleckstrin homology (PH) domains such as PLC-γ1, PLC-γ2, Akt, and Btk (see section 1.6.2). PI3K activity provides survival signals mediated largely by the activation of Akt/PKB which regulates a variety of apoptosis related proteins including Bad (Marte et al. 1997; Cox et al. 2003). PI3K is composed of a catalytic (p110α, -β,-δ) and an adaptor (p85α, p55α, p50α, p85β, p55γ) subunit.

Ras-GTP has been found to bind the PI3K p110α/β catalytic subunits and affect the production of 3' phosphoinositides (Rodriguez-Viciana et al. 1994; Rodriguez-Viciana et al. 1996; Yan et al. 1998). H-Ras has a far greater ability to activate PI3K then N- and K-Ras (Yan et al. 1998; Li et al. 2004), raising the possibility that this pathway is not prominent in lymphocytes since they lack H-Ras expression.

PLCε

PLCε contains the phospholipase C (PLC) catalytic domains in addition to a Ras GEF (CDC25) domain and two RBDs (Kelley et al. 2001; Lopez et al. 2001; Song et al. 2001). This protein, which was independently cloned by three different groups, has been shown to be activated by Ras (Kelley et al. 2001; Song et al. 2001; Song et al. 2002), Rap (Song et al. 2001; Song et al. 2002), Rho (Wing et al. 2003), and heterotrimeric G proteins (Lopez et al. 2001; Wing et al. 2001). The CDC25 domain of PLCε has been shown to activate Rap (Jin et al. 2001; Song et al. 2002), and there is also some evidence of Ras activation (Lopez et al. 2001). In addition, like other PLC family members, PLCε can generate DAG and calcium (see section 1.5.1), which in lymphocytes causes Ras activation.

p120GAP

GTPase activating proteins (Ras GAPs) (see section 1.4) inactivate Ras. p120GAP/RasGAP has been shown to have Ras GAP activity (see section 1.4), and can act as a Ras effector due to its GTP-specific binding (McCormick 1989). While the GAP activity is contained in the carboxy-terminal half of the protein, p120GAP also contains several other domains including a polyproline binding SH3, two phosphotyrosine binding

SH2 domains, a phosphoinositide binding pleckstrin homology (PH) domain, and a calcium binding C2 domain (Trahey et al. 1988). Several proteins have been shown to interact with these domains allowing for p120GAP to regulate a variety of processes downstream of Ras activation (Settleman et al. 1992; Tocque et al. 1997; Leblanc et al. 1998; Cox et al. 2003).

Ral GEFs

RalGDS (Ral GDP dissociation stimulator), Rgl1 (RalGDS–like), Rgl2, and Rgl3 are GEFs for the Ral family of Ras related small G proteins that have been shown to be Ras effectors (Hofer et al. 1994; Kikuchi et al. 1994; Spaargaren et al. 1994; Peterson et al. 1996; Wolthuis et al. 1997; Ehrhardt et al. 2001; Rodriguez-Viciana et al. 2004). Thus, Ras can control one of it's close relatives, and in doing so can mediate a wide range of effects on cell morphology, vesicle sorting/trafficking and transcription (Feig 2003).

Tiam1, RasSF1, Nore1, Rin, AF-6, Rain, IMP

Tiam1 (T lymphoma invasion and metastasis protein) is a Rac GEF that binds to H-Ras-GTP (Lambert et al. 2002). Thus, Tiam1 links two different Ras superfamilies and allows Ras signaling to induce actin reorganization. Tiam1 also possesses a PH domain, and thus can be activated by PI3K (Rameh et al. 1997). Ras promotes apoptosis through GTP-dependent interactions with the tumor suppressor, RASSF1 (Vos et al. 2000). Tumor cell lines frequently display reduced expression of RASSF1. Another closely related protein, Nore1 (novel Ras effector 1, RASSF5, RapL), also binds Ras-GTP and can heterodimerize with RASSF1 and induce apoptosis (Vavvas et al. 1998; Ortiz-Vega et al. 2002). Rin1 (Ras-interacting) is a Rab5 GEF that has been shown to interact with Ras in a GTP-dependent fashion, and can also activate the Abl tyrosine kinase (Han et al. 1995; Afar et al. 1997; Han et al. 1997; Tall et al. 2001). This is yet another link between Ras and other Ras superfamily members as well as a connection between Ras and endocytosis. AF-6 (also known as MLLT4, Afadin) has also been found to be a putative Ras effector (Kuriyama et al. 1996) that is involved in cell-cell adhesion (Asakura et al. 1999; Yokoyama et al. 2001). This protein contains 2 RBDs, a forkhead-associated domain (FHA), a DIL domain, a PDZ domain and a proline rich domain (Ponting 1995). Interaction with of AF-6 with Rap-GTP is stronger than that of Ras (Boettner et al. 2000). Thus, it is possible that *in vivo* AF-6 is predominantly a Rap effector. Rain (Ras-interacting protein) is a newly identified endomembrane associated Ras effector (Mitin et al. 2004). Little is known about this protein, however, it has a DIL domain which has been implicated in vesicle trafficking. IMP (Impedes Mitogenic signal Propagation) is a Ras effector that is proposed to modulate the threshold of Raf-Mek signaling (Matheny et al. 2004). IMP is an E3 ubiquitin ligase that upon Ras-mediated activation auto-ubiquitinates and is degraded which releases its inhibition of Raf-Mek complex formation.

1.4 Ras activation

The Ras proteins are small, 21 kDa GTPases that cycle between inactive GDP-bound and active GTP-bound forms (Figure 1-1). Ras has a high affinity for both GDP and GTP with a K_d of approximately 10^{-11} M (Feuerstein et al. 1987; Neal et al. 1988) and a slow spontaneous hydrolysis of GTP (Sweet et al. 1984), suggesting that the rapid cycling between GTP- and GDP-bound forms is assisted by other proteins. In support of the hypothesis that cellular proteins enhance Ras GTPase activity, purified Ras proteins hydrolyze GTP *in vitro* at a much slower rate then they do *in vivo* (Trahey et al. 1987). Proteins that enhance the catalytic activity of Ras have been termed Ras GTPase activating proteins (Ras GAPs). A recent review found that approximately 0.5 percent of the human genome is predicted to encode GAPs for the Ras superfamily of GTPases (Bernards 2003). This includes 14 GAPs for the classical Ras subfamily, though functional GAP activity has only been demonstrated for half of these proteins. The functional Ras GAPs can be divided into four groups: p120GAP, NF1, $\mathrm{Gap1^{m}}$ (including $\mathrm{Gap1^{m}}$, $\mathrm{GAP1^{IP4BP}}$, CAPRI), and SynGAP (including SynGAP and RasAL2) (Bernards 2003). Proteins within each of these groups have a variety of regulatory domains resulting in different mechanisms for activation including phosphorylation, calcium binding, lipid binding and protein-protein interactions (Bernards 2003; Bernards et al. 2004).

Dissociation of GDP from Ras allows for the binding of the more prevalent GTP and thus induces Ras activation. Ras guanyl nucleotide exchange factors (Ras GEFs) facilitate the dissociation of GDP from Ras. All Ras GEFs contain a conserved catalytic domain. This domain was first identified in the yeast exchange factor, CDC25 (Jones et al. 1991; Lai et al. 1993). Mammalian Ras GEFs include Sos (Sos1 and Sos2), RasGRF (RasGRF1 and RasGRF2), and RasGRP (RasGRP1, RasGRP2, RasGRP3 and RasGRP4) families. Unfortunately, we do not known the complete story of which Ras GEFs activate which Ras family members, as comprehensive analyses have not yet been performed. Nonetheless, a great deal of work has been done in order to gain a better understanding of the role of these proteins in regulating Ras.

RasGRFs are Ras activators expressed predominantly in the brain, although expression is not limited to these tissues (Chen et al. 1993; Fam et al. 1997; Font de Mora et al. 2003). Sos (Son of Sevenless) was originally identified in *Drosophila melanogaster* as a protein that functions to link the Sevenless receptor to Ras (Simon et al. 1991; Bonfini et al. 1992). Mammalian cells ubiquitously express two Sos proteins, Sos1 and Sos2 which are closely related (approximately 67% amino acid identity)(Bowtell et al. 1992; Chardin et al. 1993). Sos1 and Sos2 (collectively referred to as Sos) both have a CDC25 domain allowing for the activation of Ras, as well as a proline-rich region involved in protein-protein interactions. In addition to these domains, they also have a Dbl homology (DH) domain followed by a pleckstrin homology (PH) domain which is characteristic of GEFs specific for Rho family of small G proteins (Hoffman et al. 2002). The determination as to which GTPase Sos will activate is regulated by interactions with Grb2 and E3bl adaptor proteins (proteins with protein-protein interaction domains and no intrinsic catalytic activity) that bind in a mutually exclusive manner to the proline-rich region and allow for Ras or Rac activation respectively (Nimnual et al. 1998; Innocenti et al. 2002). In the case of Ras activation, Grb2 translocation to the plasma membrane after certain stimuli results in co-recruitment of Sos allowing it to act upon Ras. Membrane recruitment alone is sufficient to allow for Ras activation by Sos (Aronheim et al. 1994; Quilliam et al. 1994).

RasGRPs

The Ras guanyl nucleotide releasing proteins (RasGRPs) are a family of Ras GEFs that have calcium and diacylglycerol (DAG) binding domains (Figure 1-2). There are currently four mammalian RasGRPs, each of which exhibits limited tissue expression (Table 1-1).

1.4.1 RasGRP1

RasGRP1 (also known as CalDAG-GEFII) was first discovered in a screen to identify rat brain cDNAs that could cause transformation in fibroblasts expressing a mildly transforming Ras allele (Ebinu et al. 1998). Concurrently, RasGRP1 was independently cloned from a T cell cDNA library by its ability to cause transformation of NIH 3T3 cells (Tognon et al. 1998). The resulting cDNAs identified in both screens were amino- and carboxy-terminal truncated version of RasGRP1. Sequence analysis revealed several putative domains including a Ras exchange motif (REM) and a CDC25 domain that shares 50% identity with the same domain in Sos and RasGRF1 (Figure 1-2). The presence of these Ras activation domains suggested that RasGRP1 is a Ras GEF. Consistent with this hypothesis, the catalytic region of RasGRP1 was shown to induce GDP dissociation and increased GTP binding to H-Ras (Ebinu et al. 1998). As well, overexpression of RasGRP1 in fibroblasts induced elevated levels of Ras-GTP and phosphorylated Erk (Ebinu et al. 1998; Tognon et al. 1998). RasGRP1 has been shown to have the ability to activate H-Ras, N-Ras, K-Ras 4B, R-Ras, TC21 and M-Ras but not Rap1A, Rap2A or RalA (Ebinu et al. 1998; Kawasaki et al. 1998; Tognon et al. 1998; Ohba et al. 2000; Yamashita et al. 2000; Caloca et al. 2003; Guilbault et al. 2004). It is worth noting, however, that it is not known which of these interactions is the most prominent *in vivo* as these Ras activation results are from overexpression or *in vitro* mixing which could result in non-physiological activity. RasGRP1 has also been identified as an oncogene candidate based on retroviral insertion analysis of murine T cell lymphomas (Kim et al. 2003; Klinger et al. 2005).

In addition to catalytic domains involved in Ras activation, RasGRP1 also possesses regulatory domains. These include a pair of EF-hands which bind calcium and a C1 domain that has been shown to bind diacylglycerol (DAG) and DAG analogues (Ebinu et al. 1998; Lorenzo et al. 2000). The function of the EF-hands remains elusive. Despite being shown to bind calcium (Ebinu et al. 1998), the EF-hands are not required for the transforming ability of RasGRP1 (Tognon et al. 1998). Additionally, calcium at physiologically relevant concentration does not affect DAG analogue binding to the C1 domain (Lorenzo et al. 2000). However, there is some evidence that calcium can have a small effect on RasGRP1 GEF activity (Kawasaki et al. 1998), although others have found that calcium has no effect (Ehrhardt et al. 2004). RasGRP1 is the only family member for which calcium binding to the EF-hands has been demonstrated.

Despite the lack of progress in determining the function of the EF-hands, the significance of the C1 domain has been well characterized. The C1 domain was originally identified as a conserved cysteine rich region in the protein kinase C (PKC) family that is required for phorbol ester binding (Ono et al. 1989). C1 domains contain an approximately 50 amino acid conserved sequence of $Hx_{11-12}Cx_2Cx_{12-14}Cx_2Cx_4Hx_2Cx_{6-7}C$, where H is histidine, C is cysteine and x is any amino acid (Irie et al. 2004). This sequence allows for a structure that co-ordinates two zinc ions. Structurally, C1 domains have two loops with a groove between them where DAG can bind. The residues within these loops dictate whether or not a C1 domain can bind DAG (Kazanietz et al. 1995; Hurley et al. 1997; Shindo et al. 2001). PKC family members contain two tandem C1 domains while RasGRP1 contains only a single domain. Binding of the DAG analogue Phorbol 12,13-Dibutyrate (PDBu) by the RasGRP1 C1 domain was shown to be of high affinity with a K_d of 0.6-1.5 nM; this is similar to that of PKC family members (Lorenzo et al. 2000; Madani et al. 2004). In accordance with this similar affinity, the RasGRP1 C1 domain is proposed to be structurally very similar to the PKC C1 domains (Rong et al. 2002). However, PDBu binding by RasGRP1 was shown to be significantly less dependent on acidic phospholipids than PKCα, which could result in differential membrane localization *in vivo* (Lorenzo et al. 2000). As with PKC family members, some investigators have observed that prolonged Phorbol 12-myristate 13-acetate (PMA, a DAG analogue) treatment results in decreased RasGRP1 proteins levels treatment

(Rambaratsingh et al. 2003; Ehrhardt et al. 2004), although others have found this not to be the case (Stone et al. 2004). RasGRP1, much like the PKCs, is capable of binding and being activated by bryostatins and bryologues, and is inhibited by calphostin C (Lorenzo et al. 2000; Madani et al. 2004; Stone et al. 2004). Thus, the RasGRP1 C1 domain shares many similarities to those of the PKC family and is a functional DAG binding domain.

PMA induces RasGRP1 membrane translocation that is dependent upon the C1 domain. PMA-mediated transformation of cells overexpressing RasGRP1 requires the C1 domain although a C1 deletion mutant can be rescued with a lipidation signal (Ebinu et al. 1998; Tognon et al. 1998). Additionally, PMA causes increased Ras activation in cells expressing RasGRP1 (Ebinu et al. 1998). Thus, it appears that the C1 domain of RasGRP1 regulates its catalytic function by recruitment to membranes where Ras is located, reminiscent of Sos recruitment to the plasma membrane by Grb2. Interestingly, like Sos, RasGRP1 also contains a proline-rich region although the significance of this has yet to be elucidated. This proline-rich motif is not required for RasGRP1 transforming ability (Tognon et al. 1998).

RasGRP1 was initially cloned from a rat brain cDNA library and its expression in the brain has been extensively characterized (Kawasaki et al. 1998; Pierret et al. 2000; Pierret et al. 2001; Toki et al. 2001; Pierret et al. 2002). RasGRP1 was also shown to be expressed in several other tissues including thymus, spleen, lymph nodes, blood, skin and kidneys (Table 1-1) (Ebinu et al. 1998; Kawasaki et al. 1998; Tognon et al. 1998; Rambaratsingh et al. 2003). Within the lymphoid tissues, RasGRP1 has been shown to be expressed in T cells and B cells (Ebinu et al. 2000; Priatel et al. 2002). In humans, the gene encoding RasGRP1 was found to be located to 15q15, while the mouse gene is located at 2 E5 near thrombospondin (Bottorff et al. 1999).

1.4.2 RasGRP2

RasGRP2 (CalDAG-GEFI) was identified by a differential display method in an attempt to isolate cDNAs enriched in the striatum region of the brain (Kawasaki et al. 1998). Genomic sequencing of the human genomic region 11q13 had also identified this gene, termed CDC25L, although very little characterization had been performed (Kedra et al. 1997). Although the initially proposed name for RasGRP2, CalDAG-GEFI, refers to the function of its regulatory and catalytic domains, the C1 domain of this protein does not bind DAG analogues (Irie et al. 2004). Despite the lack of DAG binding, RasGRP2 has been shown to translocate from the cytoplasm to the plasma membrane upon PMA treatment (Clyde-Smith et al. 2000). The kinetics for this are much slower than for RasGRP1 (Ebinu et al. 1998; Tognon et al. 1998). While RasGRP2 lacks any obvious membrane recruitment domain, an alternatively spliced form, referred to as RasGRP2l (RasGRP2 long), was identified and found to be lipidated allowing for membrane localization (Clyde-Smith et al. 2000). The lipidation of RasGRP2l arises due to amino-terminal consensus sequences for co-translational myrisotylation and posttranslational palmitoylation located in the additional 62 amino acids contained in this splice variant. For simplicity, the originally cloned RasGRP2 will be referred to RasGRP2s (RasGRP2 short), and RasGRP2l plus RasGRP2s will be collectively referred to as the RasGRP2. Aside from amino-terminal differences, the two RasGRP2 proteins are identical and both contain REM, CDC25, EF-hands, and C1 domains (Figure 1-2).

The GEF activity of RasGRP2l differs from that of RasGRP2s. Overexpression of RasGRP2s has been shown to induce the activation of Rap1A and Rap2A but not classical Ras family members (Kawasaki et al. 1998; Clyde-Smith et al. 2000; Yamashita et al. 2000; Dupuy et al. 2001) although one study found that it was capable of activating N-Ras *in vitro* (Clyde-Smith et al. 2000). Overexpression of RasGRP2l on the other hand has been shown to activate not only Rap1 but also N-Ras and K-Ras (Clyde-Smith et al. 2000). It is, however, worth noting that RasGRP2l induces considerably less activation of these Ras proteins as compared to RasGRP1. RasGRP1 overexpression in three different cell types resulted in elevation of Ras-GTP levels from approximately 5 to 40 % in one (background to induced), and 10 to 30% in two others, while RasGRP2l overexpression in a similar assay resulted in only an increase from 3 to 7% (Ebinu et al. 1998; Kawasaki et al. 1998; Clyde-Smith et al. 2000). This could be a result of different techniques or RasGRP2l could be a poor activator of each G protein. It remains to be proven definitively which GEF activity of RasGRP2 is relevant *in vivo*.

Overexpression studies have suggested that the GEF activities of RasGRP2 are affected by calcium and DAG analogues. Calcium ionophore was shown to enhance Rap activation by both RasGRP2l and RasGRP2s, while inhibiting N-Ras activation (Kawasaki et al. 1998; Clyde-Smith et al. 2000). Since the generation of DAG by phospholipase C is accompanied by elevated calcium levels (see section 1.5.1), it is possible that Ras GEF activity is suppressed upon *in vivo* activation of RasGRP2. In cells overexpressing either form of RasGRP2, PMA increases both Ras and Rap activation, although, as mentioned previously, the C1 domain of RasGRP2 is not a functional DAG receptor.

RasGRP2s overexpression has been shown to transform cells (Dupuy et al. 2001). A viral insertion site was identified in the *RasGRP2* gene in a murine leukemia, which induced overexpression. This was also confirmed by *in vitro* overexpression studies. The addition of PMA and calcium ionophore enhanced this transformation. These stimulatory agents caused increased Rap1-GTP levels but had no effect on Ras-GTP levels after 15 minutes of stimulation. As Ras-GTP was assayed using a pan-specific antibody, GTP levels reflect all four classical Ras isoforms, provided they are expressed in these cells (Dupuy et al. 2001). These results are in direct opposition to previously published results (Clyde-Smith et al. 2000) thus further raising questions as to the GEF specificity of RasGRP2. Given the weak and conflicting nature of the evidence for Ras GEF activity and the strong evidence supporting its role as a Rap activator, RasGRP2 is likely to be a Rap-specific GEF. However, further studies are needed in order to confirm this hypothesis.

Northern blot analysis demonstrated that RasGRP2 is widely expressed, with greatest expression in heart, brain and kidney (Table 1-1) (Kedra et al. 1997; Kawasaki et al. 1998; Clyde-Smith et al. 2000). As with RasGRP1, the expression pattern of RasGRP2 within the brain has been extensively characterized (Kawasaki et al. 1998; Yamashita et al. 2000; Toki et al. 2001). RasGRP2 protein has also been found to be expressed in megakaryocytes and platelets as well as neutrophils (Eto et al. 2002; Crittenden et al. 2004). Immunohistochemical analysis suggests that mouse lymphocytes do not express RasGRP2 (Crittenden et al. 2004), while RT-PCR analysis of lymphoid cell lines suggests T cells and B cells do express RasGRP2 (Roose et al. 2005). Differential protein expression of the two splice forms has yet to be determined. In humans, the gene encoding the RasGRP2s was found to be located to 11q13, while the mouse gene is located at 19 A (Kedra et al. 1997).

1.4.3 RasGRP3

RasGRP3 (also known as KIAA0846 and CalDAG-GEFIII) was cloned by the KAZUSA human cDNA-sequencing project (Kazusa DNA Research Institute, Chiba, Japan). It was identified as a Ras GEF by database searching for cDNAs encoding proteins with putative CDC25 domains (Rebhun et al. 2000; Yamashita et al. 2000). RasGRP3 shows the broadest substrate specificity of the RasGRPs. In overexpression and *in vitro* experiments RasGRP3 has been shown to be a GEF for H-Ras, Rap1A, Rap2A, R-Ras, TC21, RalA and M-Ras (Ohba et al. 2000; Rebhun et al. 2000; Yamashita et al. 2000). There is also indirect evidence that RasGRP3 acts on N-Ras and K-Ras 4B (Oh-hora et al. 2003). Kinetic studies suggest that the activation of Rap1A by RasGRP3 is far less efficient than that induced by RasGRP2, while H-Ras activation by RasGRP3 is far more efficient than that induced by RasGRP1 (Yamashita et al. 2000). PMA has been shown to enhance RasGRP3-mediated Ras activation (Lorenzo et al. 2001). In addition, RasGRP3 has been shown to cause anchorage-independent growth of Rat1 cells, although RasGRP1 was more effective in this assay (Yamashita et al. 2000). Interestingly, RasGRP2 did not induce anchorage-independent growth in this assay (Yamashita et al. 2000) despite causing transformation in other cell types (Dupuy et al. 2001).

RasGRP3 has a similar domain structure to the other RasGRPs (Figure 1-2). The C1 domain of RasGRP3 binds PDBu with a K_d of approximately 1.5 nM indicating that it is a functional DAG binding domain (Lorenzo et al. 2001; Irie et al. 2004). RasGRP3 binding to DAG requires more anionic phospholipids (such as phosphatidylserine) than RasGRP1. This suggests there may be a difference in the membrane targeting of these two Ras GEFs upon DAG production (see 1.5.2). PMA also induced the translocation of RasGRP3 from the cytosol to the plasma membrane and Golgi (Lorenzo et al. 2001).

RasGRP3 is expressed in the brain, heart, lung, kidney, skeletal muscle, liver, and placenta as determined by reverse transcriptase-PCR and Northern blotting (Table 1-1)

(Yamashita et al. 2000). RasGRP3 has also been shown to be expressed in B cells and blood vessels and hence this may be the source of the RasGRP3 expression detected in the tissues mentioned above (Oh-hora et al. 2003; Teixeira et al. 2003; Roberts et al. 2004; Zheng et al. 2005). In humans, the gene encoding RasGRP3 was found to be located to 2p23, while the mouse gene is located at 17 E2 (Lorenzo et al. 2001).

1.4.4 RasGRP4

RasGRP4 was cloned by two separate groups. Reuther *et al.* discovered RasGRP4 in a screen for oncogenes in cytogenetically normal acute myeloid leukemias (Reuther et al. 2002). Yang *et al.* discovered RasGRP4 by sequencing cDNAs obtained from murine bone marrow derived mast cells (Yang et al. 2002). The domain structure of RasGRP4 is similar to the other RasGRPs (Figure 1-2). All four of the RasGRP family members share approximately 40-50 % identity at the amino acid level. The C1 domain of RasGRP4 binds to DAG analogues with a K_d of approximately 1.1 nM, which is similar to RasGRP1 and RasGRP3 (Irie et al. 2004). PMA was also shown to induce RasGRP4 membrane translocation (Reuther et al. 2002).

In vitro RasGRP4 acts as a GEF for H-Ras. This activity was shown to be inhibited by calcium although the levels used in this experiment (mM) were well above physiological levels (nM-uM) (Cullen et al. 2002; Yang et al. 2002). In overexpression studies RasGRP4 was transforming and caused activation of H-Ras but not Rap1A (Reuther et al. 2002). PMA treatment enhanced both transformation and Ras activation (Reuther et al. 2002; Yang et al. 2002).

RasGRP4 is expressed in blood leukocytes, lung and bone marrow, with significantly lower expression seen in several other tissues as determined by Northern blotting and reverse transcriptase-PCR (Table 1-1) (Li et al. 2002; Reuther et al. 2002; Yang et al. 2002). Most notably, RasGRP4 is not expressed in lymphocytes but rather in mast cells. The limited expression of RasGRP4 is unlike the other RasGRPs, which show more broad expression. In particular, RasGRP4 is not expressed in the brain, while the other RasGRPs are. Expression of RasGRP4 in fetal tissues is more widespread. Two RasGRP4 variants have been documented, with one having a premature translationtermination and the other a small deletion. While these transcripts are not normally present in mast cells, they are detected in mast cells derived from asthma patients (Yang et al. 2002; Li et al. 2003). In humans, the gene encoding RasGRP4 was found to be located to 19q13.1, while the mouse gene is located at 7B1 (Yang et al. 2002).

1.5 Activation of the RasGRPs

RasGRP1, RasGRP3 and RasGRP4 bind to DAG and, therefore, lipid metabolism affects the subcellular localization of these proteins. This allows DAG to act on RasGRPs in a similar manner as Grb2 acts on Sos. Additional mechanisms also exist that facilitate the recruitment of the RasGRPs to the membrane and mediate their activation.

1.5.1 Diacylglycerol metabolism

DAG does not refer to a single molecule but rather refers to a class of molecules that are closely related. DAG is composed of a glycerol backbone in which the hydroxyl groups in the *sn*-1 and *sn*-2 positions are substituted with fatty acids (FA). The FAs in the sn-2 position are highly variable while the sn-1 position is usually occupied by a saturated or monounsaturated FA (Wakelam 1998).

While DAG is a component of several signaling cascades, it is also utilized in phospholipid synthesis. However, the DAG generated by phospholipid synthesis is generally transient and would not accumulate in quantities sufficient to allow signaling to occur (Wakelam 1998). A large number of signaling cascades are capable of inducing the production of DAG by the activating phospholipase C (PLC) and phospholipase D (PLD). The phosphatidylinositol-PLC family cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to generate inositol 1,4,5-trisphosphate (IP3) and DAG. As PIP2 contains mainly polyunsaturated FA, PLC activation results in the production of polyunsaturated DAG. Additionally, IP3 production results in calcium release from the ER via IP3 receptors (Bosanac et al. 2004). The phosphatidylcholine (PC)-PLD family cleave PC to generate phosphatidic acid (PA) that is hydrolyzed to DAG by phosphatidic acid phosphatase (PAP) (Wakelam 1998). As PC contains mainly saturated FA, PLD activity produces primarily saturated PA and DAG.

The production of DAG following a variety of different signals has been shown to result in a biphasic response with PIP2 hydrolysis primarily at early time points followed by a sustained PC hydrolysis (Pessin et al. 1989; Raben et al. 1990; Pessin et al. 1991; Pettitt et al. 1993). This results in transient polyunsaturated DAG production followed by a more sustained saturated DAG level mediated by PLC and PLD respectively. The difference in DAG species produced by PLC and PLD is of great importance since PIP2, but not PC, hydrolysis results in PKC activation *in vivo* (Leach et al. 1991; Pettitt et al. 1997; Pettitt et al. 1998). Polyunsaturated DAGs are also better PKC activators *in vitro* (Marignani et al. 1996). Thus, for the RasGRP family, with the exception of RasGRP2, PLC-generated DAG is very likely the only species relevant *in vivo* due to the similarity of RasGRP C1 domains to those of the PKC family in terms of sequence and binding characteristics (Kazanietz et al. 2000; Lorenzo et al. 2000; Lorenzo et al. 2001; Shao et al. 2001; Rong et al. 2002; Irie et al. 2004; Madani et al. 2004; Stone et al. 2004).

Diacylglycerol kinases (DGKs) oppose the activity of PLC by phosphorylating DAG and generating PA. There are 9 DGK isozymes, with lymphocytes expressing DGKα and DGKζ (Yamada et al. 1989; Sanjuan et al. 2001; Zhong et al. 2002; Sanjuan et al. 2003). Both of these DGKs contain catalytic domains and two cysteine-rich domains, while DGKα also contains a C1 domain and a pair of EF-hands, DGKζ contains a nuclear localization motif overlapped by a MARCKS phosphorylation motif, a PDZ-binding motif and ankyrin repeats (Goto et al. 2004). The C1 domain and EF-hands of DGKα suggest a possible co-localization with RasGRPs that also possess these domains. Indeed RasGRP1 and DGKα have been shown to co-localize in TCR-stimulated Jurkat cells (Sanjuan et al. 2003). DGKζ has a large number of protein-protein interaction domains, and has also been found to associate with RasGRP1 (Topham et al. 2001). Overexpression of DGKζ has been shown to decrease Ras activation upon TCR stimulation as well as inhibit Ras activation induced by RasGRP1 overexpression, while DGKζ deficiency increases TCR-Ras-Erk signaling (Topham et al. 2001; Zhong et al. 2002; Zhong et al. 2003). In addition, expression of a catalytically inactive DGKα was found to increase RasGRP1 membrane association and Erk phosphorylation (Jones et al. 2002; Sanjuan et al. 2003). These results support the notion that DGKs are inhibitory to RasGRP signaling.

1.5.2 Subcellular localization of the RasGRPs

RasGRP1, RasGRP3, and RasGRP4 possess functional C1 domains, allowing for DAG-dependent membrane translocation. However, the question arises as to which membranes and membrane domains they are recruited because N-Ras and K-Ras4B have been shown to be differentially located. Several studies have identified the Golgi as an important location for Ras signaling (Chiu et al. 2002; Perez de Castro et al. 2004). The first published reports of RasGRP1 localization under overexpression conditions found cytosolic distribution in unstimulated NIH 3T3 fibroblast cells, while PMA induced translocation to plasma membrane (PM) and perinuclear structures likely to be ER and Golgi (Tognon et al. 1998). In these cells, serum induced RasGRP1 translocation to perinuclear structures. The translocation of the RasGRP1 C1 domain alone upon PMA treatment occurred in a temporal pattern with initial PM localization followed by perinuclear (Tognon et al. 1998), presumably reflecting the diffusion of the PMA which shows a similar localization pattern (Braun et al. 2005). Subsequently, a large number of studies have analyzed the subcellular localization of the RasGRPs.

When exogenously expressed in COS1 fibroblasts, RasGRP1 and RasGRP3 show Golgi localization in the presence of serum, while in the absence of serum they are cytosolic (Bivona et al. 2003; Caloca et al. 2003; Caloca et al. 2003). Similarly, in the absence of serum, RasGRP1 has also been shown to be cytosolic in DT40 chicken B cells (Caloca et al. 2003; Oh-hora et al. 2003), Jurkat T cells (Bivona et al. 2003; Carrasco et al. 2004; Perez de Castro et al. 2004), and PC12 neuroendocrine cells (Bivona et al. 2003). Perinuclear localization of endogenous or transfected RasGRP1, which many studies have identified as being Golgi and ER, has been visualized in several different cell types with various treatments including: DT40 cells treated with PMA (Caloca et al. 2003), COS1 cells treated with serum, PMA or epidermal growth factor (Bivona et al. 2003; Caloca et al. 2003; Caloca et al. 2003), Jurkat T cells treated with soluble anti-TCR antibodies (Bivona et al. 2003; Carrasco et al. 2004; Perez de Castro et al. 2004), and

PC12 cells treated with PMA or nerve growth factor (Bivona et al. 2003). It is important to note that several of these stimuli also induce PM localization. As well, certain stimuli induce only PM localization of RasGRP1 such as anti-IgM treatment of DT40 B cells (Oh-hora et al. 2003). Interestingly, stimulation of Jurkat T cells with anti-TCR antibodies can induce two different RasGRP1 translocation responses. If these antibodies are soluble then RasGRP1 moves to a perinuclear region (Bivona et al. 2003; Perez de Castro et al. 2004), but when antibodies are bead-bound RasGRP1 localizes to what appears to be the PM at the bead contact site (Carrasco et al. 2004). However, given that the Golgi polarizes towards the bead contact site as well (Depoil et al. 2005), RasGRP1 may be on the Golgi in this stimulation protocol. The bead-bound antibodies more closely reflect a physiologically relevant stimulus given that peptide-MHC complexes are expressed on the surface of antigen presenting cells. Thus, results with DT40 B cells and Jurkat T cells would suggest that in resting lymphocytes, RasGRP1 and RasGRP3 are cytosolic, and antigen receptor stimulation predominantly leads to what appears to be PM localization. Golgi localization of RasGRP1 and RasGRP3 may be important in Ras activation following stimulation of other receptors and may also be important in antigen receptor signaling.

When expressed in COS1 cells, RasGRP2l is located at the PM, while RasGRP2s is cytoplasmic (Clyde-Smith et al. 2000; Caloca et al. 2003; Caloca et al. 2003; Caloca et al. 2004). The PM localization of RasGRP2l in resting cells is likely due to myristolyation (see section 1.4.2). Despite having a non-functional C1 domain and lacking a lipidation motif, PMA treatment has been found to cause RasGRP2s translocation to the membrane fraction by some (Clyde-Smith et al. 2000) but not others (as data not shown in Caloca et al. 2004). Caloca *et al.* found that the amino terminus (first 150 amino acids) of RasGRP2s mediates binding to actin, and that activation of cytoskeletal regulators, such as Vav and Rac, mediate this association (Caloca et al. 2004). The binding of RasGRP2s with actin, which can occur *in vivo* and *in vitro*, results in the recruitment of RasGRP2s to the plasma membrane (Caloca et al. 2003; Caloca et al. 2004). The same group has also shown that RasGRP1 binds actin and that regulators of cytoskeletal rearrangement can also affect the localization of RasGRP1 and Ras activation in T cells and B cells (Caloca et al. 2003; Zugaza et al. 2004). Indeed, a constitutively active Vav or Rac can induce PM localization of a C1 domain-deficient RasGRP1 as well as induce activation of PLC (Caloca et al. 2003; Zugaza et al. 2004). This indicates that the effect of cytoskeletal rearrangements on RasGRP1 localization is both direct and indirect through actin association and enhanced PLC activity respectively. Constitutively active Vav was also found to induce RasGRP3 translocation from perinuclear to PM and punctuate perinuclear localization in COS1 cells cultured in serum (Caloca et al. 2003). Thus, actin remodeling regulates the localization of RasGRP1, RasGRP2 and RasGRP3. The effect of actin dynamics on RasGRP4 has yet to be assessed.

The subcellular localization of RasGRP4 has not been as well characterized. Subcellular fractionation and electron microscopy indicate that RasGRP4 is distributed between the PM and cytosol in splenic mast cells (Reuther et al. 2002; Li et al. 2003).

The RasGRPs, with the exception of RasGRP2l, are primarily cytosolic in unstimulated cells. A wide range of treatments are capable of inducing their translocation via DAG production or cytoskeletal rearrangements resulting in PM and/or Golgi localization depending on the stimulus and cell type.

1.5.3 Phosphorylation of the RasGRPs

In 2003, Teixeira *et al.* published a report demonstrating that RasGRP3 is expressed in Ramos B cells and that this protein is phosphorylated upon stimulation of these cells (Teixeira et al. 2003). PMA or anti-IgM treatment results in a decreased mobility of RasGRP3 in SDS-PAGE, suggestive of a post-translational modification. Phosphatase treatment inhibits the mobility shift of the protein, while phosphatase inhibitors induce it. The mobility shift coincided with activation of Ras in anti-IgM treated cells and prevention of phosphorylation using PKC inhibitors resulted in decreased Ras activation following PMA treatment or BCR stimulation. PKCθ was found to be capable of phosphorylating RasGRP3 *in vitro*, although most B cells do not co-express these two proteins. One interesting result was that anti-IgM treatment resulted only in a 2.8 fold increase in RasGRP3 phosphorylation despite 100 % of the protein shifting in mobility. This raises questions as to the exact nature of the mobility shift.

Subsequently Brodie *et al.* identified PMA-induced RasGRP3 phosphorylation in other cell types (Brodie et al. 2004). This phosphorylation occurs primarily on serine residues. A PKCδ, but not PKCα, kinase dead mutant was capable of inhibiting this phosphorylation. As well, PKCδ co-immunoprecipitates and co-localizes with RasGRP3 under conditions of overexpression. These results suggest PKCδ might be responsible for this activity. Brodie *et al.* also determined that PKCα, PKCδ, and PKCε are capable of phosphorylating RasGRP3 *in vitro*.

Further studies identified threonine 133 as a site of PKC-mediated RasGRP3 phosphorylation (Aiba et al. 2004; Zheng et al. 2005). This site is located between the REM and CDC25 domains of RasGRP3 suggesting it may affect GEF activity. Abrogation of this phosphorylation by a threonine to alanine mutation in RasGRP3 resulted in decreased Ras activation in BCR-stimulated B cells and PMA-stimulated Rat2 cells. This mutation did not affect the translocation of RasGRP3 to the PM upon anti-IgM treatment in DT40 B cells, further suggesting that this phosphorylation affects RasGRP3 GEF activity (Aiba et al. 2004). While phosphorylation of the corresponding site in RasGRP1 has been demonstrated, the biological significance is unknown, and there is also evidence for RasGRP2 and RasGRP4 phosphorylation at undefined sites (Roose et al. 2005; Zheng et al. 2005). Interestingly, the corresponding site in RasGRP4 is a proline, and PKC inhibitors do not affect PMA induced RasGRP4 signaling. There is also evidence of tyrosine phosphorylation of RasGRP3 mediated by Src in HEK-293 (human embryonic kidney) cells (Stope et al. 2004), although this has not been detected in lymphocytes.

1.6 RasGRP1 in T cells

Leukocytes, also known as white blood cells, are a critical cell population that allow us to fight off both external and internal threats to our health. Lymphocytes, a subpopulation of leukocytes, are involved in the adaptive immune response which allows for the specific clearance of pathogens unlike the less specific responses of the innate immune system (Hoebe et al. 2004). T cells are crucial components of the adaptive immune response. They function to regulate other cells in the immune system or to kill infected and diseased cells. T cells derive their name from their development in the thymus although their progenitors originate in the bone marrow. T cells are characterized by their surface expression of an antigen specific TCRαβ (or γδ), CD90 (Thy1), CD4 or CD8, CD3ε, CD27, and CD28 among others (Lai et al. 1998).

1.6.1 The T cell receptor (TCR)

The clonotypic TCRα; TCRβ heterodimers are the determinants of specificity for the TCR. They usually bind peptide antigens in the context of major histocompatibility (MHC) molecules expressed on antigen presenting cells. An antigen is any substance or molecule that can be recognized by the immune receptors. The diversity of the clonotypic antigen receptors is generated during T cell development by means of genomic recombination events (Livak et al. 2002). The result is that each T cell has unique antigen specificity, and thus clonal expansion of a given cell will result in an immune response to a specific antigen.

The TCRαβ heterodimers do not contain significant cytoplasmic tails and, as such, TCR signaling proceeds via the non-polymorphic CD3 (ε, γ, and δ) and ζ chains which associate with TCRαβ via non-covalent interactions and together form the TCR. These non-polymorphic transmembrane proteins lack any enzymatic activity and instead contain immunoreceptor tyrosine-based activation motifs (ITAMs) that facilitate downstream signaling (Pitcher et al. 2003). The ITAM amino acid consensus sequence is $YxxLx_{6-8}YxxL$, where Y is tyrosine, L is leucine, and x is any amino acid (Reth 1989).

1.6.2 T cell receptor signaling

Upon TCR binding to a peptide-MHC complex, an intricate signaling network is activated (Figure 1-3). Among the first steps following receptor activation is the phosphorylation of TCR ITAMs by Src family kinases Lck and Fyn (Straus et al. 1992; Gauen et al. 1994). This results in the recruitment and subsequent phosphorylation of

ZAP-70, a Syk family kinase whose Src homology 2 (SH2) domain binds phosphorylated ITAMs (Iwashima et al. 1994; Pawson 1995; Wange et al. 1995). Activated ZAP-70 then phosphorylates LAT, a transmembrane adaptor protein (Zhang et al. 1998). This allows for the recruitment and activation of PLC-γ1, again mediated by SH2 domain interactions with phosphotyrosine motifs.

PLC-γ1 contains X and Y catalytic domains that are required for phosphatidylinositol hydrolysis (see section 1.5.1). These two domains are linked by a series of regulatory domains including two tandem SH2 domains (SH2N and SH2C), a polyproline binding Src homology 3 (SH3) domain (Pawson 1995), and a series of phosphorylation sites. PLC-γ1 has been found to bind to LAT at phosphorylated tyrosine-132 (Zhang et al. 2000; Lin et al. 2001; Paz et al. 2001). This association has been mapped to SH2N of PLC-γ1 (Stoica et al. 1998; Irvin et al. 2000). The recruitment of PLC-γ1 to LAT allows for phosphorylation, and thus activation, by the Tec family kinases Itk and Rlk (Augustine et al. 1991; Park et al. 1991; Secrist et al. 1991; Weiss et al. 1991; Liu et al. 1998; Schaeffer et al. 1999). Interestingly, Tec family kinases are also involved in actin remodeling (Finkelstein et al. 2004) which has been shown to affect RasGRP1 and RasGRP2 localization and activation (see section 1.5.2). The SH3 domain of PLC-γ1 has been shown to mediate association with the adaptor protein SLP-76 (Yablonski et al. 2001). This association is required for PLC-γ1 activation, likely due to SLP-76 association with the adaptor Gads, which is associated with phosphorylated LAT and would thus increase PLC-γ1 affinity for LAT and thus further assist in maintaining membrane proximity (Yablonski et al. 2001). PLC-γ1 also contains a carboxy terminal C2 domain, a amino-terminal PH domain and a split PH domain located on either side of the SH domains. C2 domains were originally identified in PKC family members where they bind calcium (Kaibuchi et al. 1989). PH domains are functionally diverse and in the case of PLC-γ1 have been found to mediate binding to phosphatidylinositol 3,4,5-trisphosphate (PIP3), a product of PI3K signaling (Falasca et al. 1998). It has been shown that the PLC-γ1 split PH domain can function in mediating protein-protein interactions (van Rossum et al. 2005). Thus, TCR signaling results in LAT phosphorylation, which induces membrane recruitment of PLC-γ1 allowing it to be phosphorylated and activated. PLC-γ1 can then cleave PIP2 to generate IP3 and DAG.

IP3 production results in calcium release from the ER, and then subsequently induces release from extracellular stores via calcium release activated calcium (CRAC) channels at the PM (Randriamampita et al. 2004). The calcium released is involved in the activation of a large number of proteins including the conventional protein kinase C (cPKC) family and calcineurin. Activation of the serine/threonine phosphatase calcineurin, also known as PP2B, by calcium-calmodulin results in dephosphorylation of the transcription factor NFAT (Klee et al. 1998; Crabtree et al. 2002). This unmasks a nuclear localization sequence in NFAT allowing it to translocate to the nucleus. Once in the nucleus, NFAT can interact with AP-1 to activate the transcription of the IL-2 gene (see section 1.6.4).

PLC-γ1 activity also results in the production of DAG. Many proteins possess C1 domains which allow them to bind to DAG, and thus be recruited to membranes upon PLC-γ1 activation (Brose et al. 2002; Kazanietz 2002; Yang et al. 2003; Springett et al. 2004). Among the C1 domain containing proteins expressed in T cells are the conventional PKCs (cPKC, PKCα and β), the novel PKCs (nPKC, PKC δ, ε, η and θ) (Baier 2003), and RasGRP1 (Ebinu et al. 2000). cPKCs are calcium and DAG regulated serine/threonine kinases while nPKCs are DAG but not calcium regulated. Members of a third family of PKCs, the atypical PKCs (aPKC), are also expressed in T cells but they do not bind DAG (Baier 2003). PKCθ has been shown to be necessary for TCR ligation-induced IL-2 production (Werlen et al. 1998; Ghaffari-Tabrizi et al. 1999), as well as being involved in the activation of Jnk (Werlen et al. 1998; Ghaffari-Tabrizi et al. 1999), NF-κB (Bauer et al. 2000; Coudronniere et al. 2000; Lin et al. 2000; Sun et al. 2000; Bauer et al. 2001; Li et al. 2005) and NFAT (Bauer et al. 2000). PKCθ has been found to be unique among the PKCs in its localization to the mature central supramolecular activation cluster (SMAC) upon TCR-ligation (Monks et al. 1997; Monks et al. 1998; Grakoui et al. 1999). The SMAC refers to the segregation of signaling and adhesion molecules into higher order clusters at the T cell-antigen presenting cell contact site (Kupfer et al. 2003). Interestingly, in T cells stimulated with bead-bound anti-TCR RasGRP1 localizes to the T cell-bead contact site (Carrasco et al. 2004) although the localization of this protein within the SMAC has not been determined. Another similarity between PKCθ and RasGRP1 is that Vav can regulate the membrane translocation of both of these proteins (Kong et al. 1998; Hehner et al. 2000; Villalba et al. 2000; Villalba et al. 2002).

The first study of Ras activation following TCR-stimulation was performed by Downward *et al.* 15 years ago (Downward et al. 1990). They found that stimulation of human peripheral blood T cells with a CD3-specific antibody resulted in the accumulation of Ras-GTP. They observed a similar result when they stimulated these cells with phorbol dibutyrate (PDBu), a DAG analogue. Downward *et al.* found that permeabilized T cells have a high basal Ras GEF activity. They also found that PDBu treatment caused a six-fold reduction in Ras-GTP hydrolysis. Thus, since PDBu treatment alone can reduce the rate at which GTP bound by Ras is hydrolyzed, Downward *et al.* hypothesized that TCR stimulation activates an unknown nPKC family member that then regulates Ras GAP activity. This regulation would have to be indirect since, in a subsequent study, this same group observed that the two Ras GAPs expressed in these cells, p120GAP and NF1, are not phosphorylated upon PDBu or anti-TCR treatment (Izquierdo et al. 1992). In this study, they also determined that the inhibition of PKC activity using a pseudosubstrate peptide had little effect on TCR-stimulation induced Ras activation although it did inhibit the activation of this small G protein induced by PDBu. These results suggest that either there is a redundant, non-PKC pathway for Ras activation or that the PKC-mediated Ras activation plays little or no role in linking TCR to Ras. The mechanism of the proposed GTPase regulation was not uncovered.

Grb2 has been shown to associate with LAT upon TCR stimulation, providing a mechanism for Sos recruitment to the PM where it can activate Ras (Sieh et al. 1994; Nel et al. 1995; Zhang et al. 1998). Additionally, it has been demonstrated that upon TCR stimulation the adaptor protein Shc can associate with the TCR, become phosphorylated, and recruit Grb2-Sos complexes (Ravichandran et al. 1993; Li et al. 1996). However, similar studies found that this complex does not form (Gupta et al. 1994; Nel et al. 1995). Thus, it appears that Ras activation upon TCR-ligation can occur via Grb2-Sos recruitment to the membrane by LAT or possibly Shc under certain circumstances.

Despite the results supporting Sos-mediated Ras activation upon TCR stimulation, the ability of DAG analogues to induce Ras activation cannot be explained by this model (Downward et al. 1990; Izquierdo et al. 1992). Additionally, reconstitution of LAT deficient Jurkat T cells with a LAT mutant that cannot bind PLC-γ1 but still retains Grb2 binding greatly reduces Erk activation upon TCR stimulation (Zhang et al. 2000). Similarly, in peripheral T cells from LAT knock-in mice expressing a LAT mutant deficient in PLC-γ1-binding, TCR-Erk signaling was diminished (Sommers et al. 2002). Interestingly, the Grb2 binding site on LAT is the same as the closely related Gads (Zhang et al. 2000) which has been shown to be necessary for PLC-γ1 binding to LAT via the association of these proteins with another adaptor, SLP-76 (Yablonski et al. 2001). Thus, there is evidence for two distinct TCR-Ras pathways (LAT (or Shc)-Grb2-Sos, and LAT-PLC-γ1-RasGRP1) in T cells.

Shortly after the cloning of RasGRP1, evidence was presented that identified this protein as the missing link between PLC-γ1 and Ras activation in T cells (Ebinu et al. 2000). TCR stimulation results in RasGRP1 translocation to the membrane. Immunodepletion of RasGRP1 from the membrane fraction of TCR-stimulated Jurkat T cell lysates abolishes the Ras GEF activity of these fractions. As well, RasGRP1 overexpression increases TCR-Ras-Erk signaling. Thymic T cells from RasGRP1-deficient mice lack PMA-induced Ras and Erk activation as well as TCR stimulation-mediated Erk activation (Dower et al. 2000; Priatel et al. 2002). These results suggest TCR-PLC-γ1-RasGRP1 is the primary pathway for Ras-Erk activation in T cells. Interestingly, thymic T cells from mice that are heterozygous for a *Grb2* null mutation (homozygous mice are embryonic lethal) display reduced TCR-Ras signaling despite having normal TCR-ligation induced Erk activation (Gong et al. 2001). These T cells also display reduced TCR-Jnk and TCR-p38 signaling. Paradoxically, thymocytes in which Ras signaling is inhibited by the expression of a dominant negative mutant, Ras^{N17}, have normal Jnk activation upon TCR-stimulation but defective Erk activation suggesting that the decreased Jnk activation in $Grb2^{+/-}$ T cells is Ras-independent (Gong et al. 2001). This is in contrast to results from Jurkat human T cells where expression of dominant negative Ras resulted in the inhibition of both Erk and Jnk activation following TCR stimulation (Izquierdo et al. 1993; Faris et al. 1996). There is also evidence that the threshold for Erk activation is lower than that of Jnk (Gong et al. 2001). Thus, it is possible that the normal Erk activation in *Grb2*$^{+/-}$ T cells is a result of maximal activation even at lower levels of Ras-GTP while decreased Jnk and p38 activity are due to a defect in a Grb2 pathway that does not utilize Ras. Thus, it appears that there exist two different Ras GEFs that function in TCR signaling, Sos and RasGRP1.

1.6.3 T cell development

The mechanism by which Sos and RasGRP1 are differentially activated is not fully understood but the study of T cell development has provided some important clues. T cell precursors from the bone marrow enter the thymus and begin a process of development that is dependent on signals from the TCR (Figure 1-4) (von Boehmer et al. 1997). TCRα and TCRβ, the specificity determinants of the TCR, must undergo genomic rearrangements in order to generate diversity. The TCRβ chain undergoes genomic recombination before TCRα. Upon successful rearrangement, a surrogate-TCRα allows for the surface expression of TCRβ, which then induces signals that allow for the expression of the co-receptors CD4 and CD8. This is the double negative (DN, CD4^{-}CD8^{-}) to double positive (DP, CD4^{+}CD8^{+}) transition. At this point TCRα rearrangement occurs and, if productive, the encoded protein is expressed on the surface along with TCRβ. The specificity of the TCR (TCRα and TCRβ combined) then determines the next step in development. In an immune response, the TCR must recognize peptide in the context of self-MHC. Thus, it is desirable at this stage for T cells to develop that can partially recognize MHC on thymic epithelial cells but do not have high affinity binding to self-peptides. The new TCR can have no affinity, moderate affinity, or high affinity for MHC-self peptide, which results in death by neglect, positive selection, or negative selection respectively (Figure 1-4). If cells are positively selected expression of either CD4 or CD8 is lost, generating CD8 or CD4 single positive (SP) thymocytes respectively. Thus, development proceeds through DN-DP-SP in terms of CD4 and CD8 expression.

T cell development depends on pre-TCR and then TCR signaling. These signaling pathways appear to resemble closely those of mature T cells, as mutations in

Lck (Molina et al. 1992), ZAP-70 (Negishi et al. 1995), LAT (Zhang et al. 1999), SLP-76 (Clements et al. 1998), RasGRP1 (Dower et al. 2000; Priatel et al. 2002), Grb2 (Gong et al. 2001), Ras (Swan et al. 1995), Raf (O'Shea et al. 1996), Mek (Crompton et al. 1996), or Erk (Sharp et al. 1997; Pages et al. 1999; Sharp et al. 1999) results in defective development. Therefore, Ras activation is critical for T cell development and both RasGRP1 and Grb2-Sos pathways appear to be important.

Inhibition of Ras activation in T cells, due to the transgenic expression of Ras^{N17} under the Lck promoter resulted in defective positive selection, but normal negative selection (Swan et al. 1995). Thus, Ras does not mediate high affinity negative selection although it does mediate lower affinity positive selection. As well, T cells from Ras^{N17} transgenic mice have defective TCR ligation-induced proliferation and Erk activation (Swan et al. 1995; Gong et al. 2001). Conversely, expression of an active Ras, Ras^{V12}, can induce the DN-DP transition even in the absence of TCRβ rearrangement (Swat et al. 1996; Gartner et al. 1999). Similar results were found with the use of Raf (O'Shea et al. 1996; Iritani et al. 1999) or Mek (Alberola-Ila et al. 1995; Crompton et al. 1996) active and inhibitory mutants.

In *Rasgrp1*$^{-/-}$ mice, as with Ras^{N17} transgenic mice, positive selection is defective while negative selection is normal leading to reduced CD4/CD8 SP T cells (Dower et al. 2000; Priatel et al. 2002; Layer et al. 2003). RasGRP1 overexpression induces DN-DP transition in the absence of TCRβ rearrangement (Norment et al. 2003) which is similar to what is observed in Ras^{V12} transgenic mice. In *Grb2*$^{+/-}$ mice, thymocytes have normal positive selection and defective negative selection (Eto et al. 2002). While the similarity of RasGRP1 and Ras transgenic mice suggest that RasGRP1 alone is the relevant *in vivo* Ras activator in thymocytes, it is important to note that the Grb2 deficiency is only haploid. It has been proposed, based on the results mentioned above, that weak stimulation of the TCR results in RasGRP1-mediated Ras activation and positive selection while a strong stimulation results in both RasGRP1 and Grb2-Sos-mediated Ras activation and negative selection (Gong et al. 2001; Yun et al. 2001). In terms of the Mapk response, positive selection results in Erk activation while negative selection results in Erk, Jnk, and p38 activation (Figure 1-4).

1.6.4 Ras-mediated T cell responses

The activation of Ras in mature T cells results in numerous cellular changes. Ras signaling is required for TCR ligation-induced proliferation and cytokine production. One of the best-studied cytokines produced upon T cell activation is IL-2. Upon TCR ligation, the Ras-Raf-Mek-Erk pathway is activated (Izquierdo et al. 1993; Jorritsma et al. 2003). Erk then phosphorylates and activates several different proteins including the transcription factor Elk-1 (Genot et al. 1996). Elk-1, in combination with other transcription factors, induces the expression of several genes including the transcription factor *c-fos* (Whitmarsh et al. 1996). c-Fos heterodimerizes with c-Jun to form the AP-1 transcription factor and, in co-operation with other transcription factors, induces the transcription of a number of genes. IL-2 transcription requires the activation of both AP-1 and NFAT, a family of transcription factors that are activated by the calcium-calcineurin pathway (Jain et al. 1993). Expression of a constitutively active Ras has been shown to result in increased IL-2 transcription (Baldari et al. 1992), while a dominant negative Ras decreases the production of this cytokine (Yamashita et al. 1999). Alterations in Ras-Erk-AP-1 signaling have also been shown to affect the production of numerous other cytokines including IL-3, IL-4, IL-6, IL-5, IL-10, IL-13, GM-CSF, and IFN-γ (Dumont et al. 1998; Egerton et al. 1998; Yamashita et al. 1999). Thus, Ras activation results in the production of a large variety of cytokines. The specific repertoire produced by activation of this small G protein depends on the activation of other transcription factors that co-operate with AP-1.

The cytokines that are secreted by a CD4$^+$ T cell allow it to be defined generally as either Th1 or Th2 (Mosmann et al. 1986). Th1 cells are known for their secretion of IFNγ and IL-2, while Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Glimcher et al. 2000). Naïve T cells become Th1 or Th2 in response to stimulus. Using TCR transgenic CD4$^+$ T cells, it has been shown that a strong TCR stimulus induces robust and sustained Erk activation leading to a Th1 phenotype, while a weak stimulus induces only transient Erk activation and a Th2 phenotype (Jorritsma et al. 2003). This study also showed that if Erk activation was inhibited in conjunction with a strong TCR stimulation, a Th2 phenotype would occur. Under these circumstances, there was an increase in AP-1 composed of Jun-Jun homodimers rather than Fos-Jun heterodimers. This increased level of Jun-Jun homodimers is also seen in Th2 cells, with Th1 cells primarily having Fos-Jun heterodimers (Rincon et al. 1997; Jorritsma et al. 2003). This altered composition of AP-1 is known to result in altered DNA binding (Rooney et al. 1995). Interestingly, RasGRP1-deficient T cells which lack TCR stimulation-induced Erk activation, have been found to be deficient in TCR stimulation induced IL-2 and IFNγ production yet produce excessive amounts of IL-4 (Layer et al. 2003), while RasGRP1 overexpressing Jurkat T cells produce greater levels of IL-2 upon PMA-stimulation than wildtype cells (Ebinu et al. 2000). These cytokine secretion results demonstrate that $CD4^+$ T cells from *Rasgrp1*$^{-/-}$ mice have a Th2 polarization, which is similar to that which occurs in T cell when Erk is inhibited. However, there have been studies that conflict with the notion that weak Erk activation leads to Th2 and strong activation leads to Th1. For instance, T cells from a Ras^{N17} transgenic mouse have completely inhibited TCR-Erk signaling yet are Th1 polarized (Yamashita et al. 1999). However, in this study several crucial phenotypes of the Ras^{N17} transgenic mice, such as the effect of the Ras^{N17} transgene on T cell development, differ from previously published results (Swan et al. 1995) which raises questions as to whether the conclusions of each study are valid and if the transgene expression is constant in these mice.

In addition to cytokine production, Ras activation leads to the proliferation of T cells. Expression of a dominant negative Ras or treatment with the Mek inhibitor PD98059 have been shown to inhibit TCR ligation induced proliferation (Swan et al. 1995; Sharp et al. 1997; Dumont et al. 1998). Similarly, Erk1 deficient T cells display decreased TCR ligation-induced proliferation (Pages et al. 1999). Interestingly, Grb2 heterozygous T cells have normal proliferative responses (Gong et al. 2001), while RasGRP1 deficient T cells have defective proliferation, which further supports the notion that RasGRP1 activates the Ras-Erk pathway in these cells (Dower et al. 2000; Priatel et al. 2002; Layer et al. 2003). Additionally, RasGRP1 overexpression lowers the threshold for TCR ligation-mediated proliferation (Norment et al. 2003).

1.6.5 Ras and T cell anergy

The importance of Ras signaling in T cells is further highlighted by the status of this pathway in anergic T cells. Anergy is a state of T cell unresponsiveness to TCR stimulation and is thought to be a mechanism for inactivation of auto-reactive T cells after they have left the thymus. This anergic state can be achieved by stimulating naïve T cells through the TCR in the absence of a co-stimulatory signal. In anergic T cells, stimulation through the TCR fails to induce activation of Ras and Erk (Fields et al. 1996). However, in these anergic cells, TCR stimulation does lead to Grb2 binding to phosphorylated Shc, suggesting that the Grb2-Sos pathway is not affected in these cells. Expression of a constitutively active Ras does not prevent anergy (Crespi et al. 2002), and thus Ras-Erk is not the only pathway inhibited in anergic T cells. PMA-induced Ras activation is normal in anergic cells indicating that the block in Ras activation occurs at or upstream of DAG responsive proteins (Li et al. 1996). Calcium-Calcineurin-NFAT signaling is normal in anergic cells (Li et al. 1996) and NFAT1-deficient T cells are resistant to anergy suggesting that NFAT1 activates the transcription of anergy-inducing genes (Macian et al. 2002). When anergy specific gene expression was assessed, it was found that several transcripts are specifically upregulated in anergic cells. Among these upregulated genes is DGKα (Macian et al. 2002). This protein has been found to inhibit RasGRP1 membrane translocation as well as Erk activation following TCR ligation (see section 1.5.1)(Jones et al. 2002; Sanjuan et al. 2003). Another DGK family member, DGKζ, has also been shown to inhibit TCR-RasGRP1-Ras-Erk signaling and thus could also play a role in anergy (Topham et al. 2001; Zhong et al. 2002; Zhong et al. 2003). In anergic cells, increased expression of DGKs prevents PLC-γ1-induced activation of RasGRP1, thus inhibiting Ras activation upon TCR stimulation. DGKs also inhibit the PKC family, which explains why Ras activation is not sufficient to rescue the defect. Similarly, the ability of PMA to bypass anergy is due to the inability of DGKs metabolize this compound.

1.6.6 RasGRP1 deficiency

RasGRP1 deficient mice are grossly normal, fertile, and are born in normal Mendelian ratios. However, loss of RasGRP1 expression results in a T cell development deficiency at the DP-SP stage, with defective positive selection and normal negative selection of thymocytes (see section 1.6.3). *Rasgrp1*$^{-/-}$ thymocytes were shown to be defective in TCR ligation- and PMA-induced activation of Ras-Erk signaling (Dower et al. 2000; Priatel et al. 2002). The developmental defect in *Rasgrp1*$^{-/-}$ mice results in a decreased number of T cells leaving the thymus and thus a decreased peripheral T cell population in young mice.

Layer *et al.* found that aged RasGRP1 deficient mice develop a paradoxical late-onset autoimmune disorder typified by enlarged spleens, tissue lymphocyte infiltration, increased serum immunoglobulin levels, and anti-nuclear autoantibodies (Layer et al. 2003). This autoimmunity has been characterized as lupus-like due to the antinuclear antibodies, which are also present in patients with systemic lupus erythematosus (SLE). SLE is an autoimmune disorder that can affect most organs of the body with autoantibody deposition and lymphocyte infiltration causing tissue damage. The etiology of lupus has yet to be determined. Despite the similarities of the autoimmunity seen in SLE and *Rasgrp1*$^{-/-}$ mice, human SLE is a polygenic disease in which multiple loci contribute along with environmental factors. None of the identified SLE susceptibility loci co-localize with the gene encoding RasGRP1 in either humans or mouse models of SLE (Bottorff et al. 1999; Nguyen et al. 2002; Alarcon-Riquelme et al. 2003; Tsao 2003; Santiago-Raber et al. 2004). However, it has been shown that T cells from SLE patients display reduced TCR-Erk-AP1 signaling although PMA-Erk signaling is normal (Cedeno et al. 2003). This result indicates that a common signaling defect may exist between SLE patients and *Rasgrp1*$^{-/-}$ mice although the normal PMA-Erk signaling in these patients indicates the deficiency is not at the level of RasGRP1. Murine models of drug-induced SLE have also been found to have defective Erk activation and Mek inhibition was sufficient to induce the generation of anti-dsDNA antibodies (Deng et al. 2003). Despite the lack of evidence for genetic linkage to SLE, it is possible that a small subpopulation of patients have mutations in the *RasGRP1* gene that have yet to be identified.

Much like SLE, the precise cause of late-onset autoimmunity in *Rasgrp1*$^{-/-}$ mice has yet to be fully elucidated. Developmental defects in *Rasgrp1*$^{-/-}$ mice result in a reduced splenic T cell population in young mice. Splenic T cells from these mice have greatly reduced proliferation in response to anti-CD3 or PMA plus ionomycin (Priatel et al. 2002; Layer et al. 2003). Despite these defects, *Rasgrp1*$^{-/-}$ mice develop splenomegaly and autoimmunity. There are several possible mechanisms for this development. T cells from aged *Rasgrp1*$^{-/-}$ mice were found to be more autoreactive which could lead to autoimmunity, although this autoimmune population was not increased in young mutant mice (Layer et al. 2003).

The requirement for T cells in the generation of autoimmunity in *Rasgrp1*$^{-/-}$ mice has also been investigated. CD4^{+} T cells from these mice were adoptively transferred into *Rag1*$^{-/-}$ mice and found to induce splenomegaly, suggesting that these cells are responsible for generating autoimmunity (Layer et al. 2003). However, as the production of autoantibodies was not assessed in these experiments, due to the absence of B cells, this study is not conclusive. Splenic T cells in *Rasgrp1*$^{-/-}$ mice are predominantly CD4^{+}, with a greatly reduced CD8^{+} population. These CD4^{+} T cells express CD44, which is characteristic of memory T cells (Sprent et al. 2002). When stimulated through the TCR for three days, these T cells secrete greatly reduced levels of the TH1 cytokines IFNγ and IL-2 as compared to wildtype (Layer et al. 2003). However, T cells from aged *Rasgrp1*$^{-/-}$ mice stimulated in this manner secrete greatly elevated levels of IL-4, a TH2 cytokine. These CD4^{+} T cells also display reduced activation-induced cell death (AICD) and a decreased ability to kill activated B cells, both of which are Fas-mediated processes. Fas is a surface receptor expressed on activated B cells and many other cell types that when bound by FasL expressed on activated T cells induces an apoptosis inducing signaling cascade. Since RasGRP1 deficient B cells display normal Fas-mediated death in response to wildtype T cells, it is possible that the autoimmunity derives from a decreased death of both activated T cells and B cells in *Rasgrp1*$^{-/-}$ mice. This is analogous to *lpr* mutant mice, which have a deficiency in Fas expression and also develop autoimmunity (Santiago-Raber et al. 2004). However, decreased death alone is unlikely to be sufficient to generate splenomegaly, as the proliferation defect of *Rasgrp1*$^{-/-}$ T cells is quite substantial. It is important to note that the proliferative response of

Rasgrp1$^{-/-}$ T cells can be partially rescued by the addition of IL-2 (Priatel et al. 2002; Layer et al. 2003).

One possible contribution to the enlargement of the T cell population is homeostatic proliferation, which is the compensatory expansion of a population in order to achieve a homeostatic number of cells. King *et al.* found that homeostatic proliferation following lymphopenia results in autoimmunity and that this proliferation was cytokine-mediated (King et al. 2004). They showed that increasing T cell numbers by cell transfer from mice of the same genotype was sufficient to prevent autoimmunity and, thus, that the proliferative pressure in these mice is what causes the autoimmunity. The lymphopenia in young *Rasgrp1*$^{-/-}$ mice caused by defective T cell development may contribute to autoimmunity. Indeed, several other transgenic mice with T cell development deficiencies due to TCR signaling defects develop late-onset autoimmunity (Aguado et al. 2002; Sommers et al. 2002; King 2004). One of these examples is *LAT*^{Y136F} knock-in mice which express a form of LAT that is incapable of binding to PLC-γ1 (Aguado et al. 2002; Sommers et al. 2002) and presumably cannot activate RasGRP1. T cells from these *LAT*^{Y136F} mice display reduced proliferation, increased IL-4 production, and elevated CD44 expression much like *Rasgrp1*$^{-/-}$ T cells. Since autoreactive B cells can survive negative selection processes when sufficient T cell help is present (Fazekas de St Groth et al. 1997; Sater et al. 1998), it is possible that the production of autoreactive T cells in *Rasgrp1*$^{-/-}$ mice is sufficient to generate autoimmunity.

The late-onset autoimmunity seen in RasGRP1-deficient mice could be due to increased autoreactivity of T cells, decreased T cell-mediated killing and activation induced cell death, altered cytokine production, homeostatic proliferation or more likely a combination of these factors. The autoantibodies produced in *Rasgrp1*$^{-/-}$ mice are proposed to be a normal response to elevated T cell signals including those specific for self-antigens. B cells from these autoimmune mice display normal proliferative responses and Fas-mediated killing. Thus, it is presumed that RasGRP3 expression in these cells allows for normal B cell responses.

1.7 Ras activation in B cells

B cells, like T cells, are involved in the adaptive immune response. B cells derive their name from their development in the bursa of Fabricius in birds. In humans and mice B cells develop in the bone marrow and continue to develop once they enter the spleen. B cells fight off pathogens by secreting antibodies, an effector mechanism that is unique to B cells. These antibodies bind to pathogens and target them for complement-mediated lysis or phagocytosis. Like T cells, B cells possess a clonotypic antigen receptor, termed the B cell receptor (BCR). This receptor contains a variable region which during B cell development is rearranged in a similar manner to the TCR, thus allowing for antigen specific B cells. Unlike the TCR, the BCR does not bind to its antigen in the context of a host binding protein but rather can bind to the antigen directly. Upon encountering antigen, a B cell can be stimulated to secrete antibodies that are an alternately spliced soluble form of the BCR. B cells are characterized by their expression of the BCR (specific isotype varies, see below), B220 (CD45R), and CD19 although the expression of these proteins is altered upon cell activation (Lai et al. 1998).

1.7.1 The B cell Receptor (BCR)

The BCR shares a great deal of similarity with the TCR. While in T cells antigen specificity is determined by the variable regions of the TCR$\alpha\beta$ heterodimer, B cells utilize the variable regions of the heavy and light chain. An immunoglobulin molecule is composed of two heavy chains and two light chains, and thus has two antigen binding sites. One unique feature of the BCR is the ability of the heavy chain to class switch from IgM/IgD to IgG3, IgG1, IgG2b, IgG2a, IgE or IgA immunoglobulin isotypes in mice. However, the variable region of the heavy chain remains the same and thus the specificity of the BCR does not change. The structural differences between immunoglobulin isotypes are located in their heavy chain constant regions, with variations in numbers of constant domains, presence of a hinge region, disulfide bonds between heavy chains and glycosylation. The process of class switching involves genomic recombination resulting in the deletion of a DNA fragment encompassing the constant regions of at least one isotype. The remaining 3' constant region is then expressed. In addition to class switch recombination, alternative splicing of the heavy chain allows for each of the immunoglobulin isotypes being either membrane bound or secreted. The light chain of the BCR does not class switch but there are two different light chains that can be expressed, κ and λ. Only one light chain is expressed in most cases, and in mice this is usually the κ chain.

Much like the TCR, the BCR consists of an antigen-binding membrane-bound immunoglobulin and a non-covalently associated Igα-Igβ heterodimer that serves to transmit signals. Igα (CD79a) and Igβ (CD79b) each contain a single ITAM motif, allowing for a similar signaling mechanism as is seen in T cells.

1.7.2 B cell receptor signaling to Ras and Erk

Ligation of the BCR by antigen binding leads to Igα-Igβ ITAM tyrosine phosphorylation by Lyn and other Src family kinases (Figure 1-5) (Kurosaki 1999; Dal Porto et al. 2004). ITAM phosphorylation allows for SH2 domain-mediated recruitment of Syk to the BCR, followed by its phosphorylation and activation (Kurosaki et al. 1994; Kurosaki et al. 1995). Btk , a Tec family kinase, is phosphorylated and activated by Lyn and Syk (Kurosaki et al. 2000). This phosphorylation requires Btk to first be recruited to the PM via the activity of PI3K, a lipid kinase. PI3K is activated by Lyn and Syk through the SH2 domain of PI3K binding to proteins containing phosphorylated YXXM motifs such as CD19 (Buhl et al. 1999; Wang et al. 2002; Dal Porto et al. 2004). Active PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 is bound by proteins with PH domains including Btk and PLC-γ2 which allow these proteins to be recruited to the membrane (Falasca et al. 1998; Dowler et al. 1999; Saito et al. 2001).

Active Syk also phosphorylates BLNK (B cell linker, also known as SLP-65). BLNK is an adaptor protein that, when phosphorylated, is bound by the SH2 domains of several proteins including Btk and PLC-γ2 (Fu et al. 1998; Hashimoto et al. 1999; Ishiai et al. 1999; Chiu et al. 2002). This association allows for Btk to phosphorylate and activate PLC-γ2, causing it to cleave PIP2 and generate DAG and IP3 (Hashimoto et al.

1999; Ishiai et al. 1999; Chiu et al. 2002). While both Syk and Btk can phosphorylate PLC-γ2, Syk appears to be required for initial activation and Btk more for sustained activation of this lipase (Fluckiger et al. 1998). The IP3 produced by PLC-γ2 activity causes calcium release from the ER via IP3 receptors (Sugawara et al. 1997). The rise in calcium then causes calcium release from extracellular stores via store operated channels (SOCS) by a process termed capacitive calcium entry (Spassova et al. 2004). Much like in T cells, increased calcium levels leads to activation of the calcineurin-NFAT pathway, thus causing alterations in transcription (Choi et al. 1994; Venkataraman et al. 1994; Sugawara et al. 1997).

Evidence exists for Ras GAP regulation in B cells (Gold et al. 1993). However, most studies have focused on Ras GEF-meditated Ras activation in B cells. Upon BCR stimulation, N-Ras, K-Ras, Raf, Mek and Erk are activated in *ex vivo* B cells and B cell lines from a variety of species (Gold et al. 1992; Tordai et al. 1994; Kashiwada et al. 1996; Li et al. 1996; Sutherland et al. 1996; Purkerson et al. 1998; Ehrhardt et al. 2004). In DT40 chicken B cells Syk and PLC-γ2 are required for BCR-Ras-Erk signaling, while Btk, BLNK and Grb2 have been found to be necessary for maximal signaling to Erk (Hashimoto et al. 1998; Jiang et al. 1998; Ishiai et al. 1999). Shc, which has been shown to bind the BCR, Syk and Grb2 upon BCR stimulation (Nagai et al. 1995), is dispensable for Erk activation in DT40 cells (Hashimoto et al. 1998). Thus, at least in DT40 cells, the Grb2-Sos pathway is not required for Ras-Erk activation and is not dependent upon Shc binding. In primary murine B cells PLC-γ2 is required for BCR-Erk signaling (Bell et al. 2004). Additionally, the DAG analogue PMA has been shown to induce Ras and Erk activation and pretreatment of cells overnight with PMA blocks BCR-Erk signaling, implicating PKC isoforms in Ras activation (Gold et al. 1992; Tordai et al. 1994; Li et al. 1996; Jiang et al. 1998; Ehrhardt et al. 2004). Thus, there appear to be two pathways mediating Ras activation upon BCR stimulation, a Grb2-Sos pathway and a PLC-γ2-DAG pathway. However, given the complete lack of Ras-Erk activation in the absence of PLC-γ2 expression, this pathway is likely the predominant one. Although RasGRP1 is expressed in B cells, the B cell population is apparently normal in RasGRP1 deficient mice suggesting that another Ras GEF, possibly RasGRP3, functions to activate Ras upon BCR ligation (Dower et al. 2000; Teixeira et al. 2003).

1.7.3 The role of Ras in B cell development

B cell development begins in the bone marrow (BM) where the heavy chain and the light chain are rearranged and the BCR is expressed for the first time. Following development in the BM, B cells migrate to the spleen where they complete their maturation (Figure 1-6). These developmental processes require signaling through a variety of receptors including the pre-BCR and BCR. The pre-BCR contains the rearranged heavy chain and a surrogate light chain. Expression of this receptor induces signaling events and signifies that a functional heavy chain has been produced and thus the light chain can be rearranged. Once a functional light chain is expressed, BCR signaling once again allows for development to proceed via positive selection or for negative selection to remove self-reactive B cells. Signaling through the BCR during development is similar to that in mature cells and deficiencies in the proteins involved in BCR signaling often result in blocks in B cell development (Kurosaki 1999; Fruman et al. 2000; Marshall et al. 2000).

Expression of a B cell-specific dominant negative Ras (Ras^{N17}) causes arrest of B cell development at the pro-B cell stage prior to the expression of the pre-BCR (Iritani et al. 1997). Such an early block in B cell development in these mice likely signifies that an essential, non-BCR pathway is inhibited by expression of this mutant protein. IL-7 receptor signaling has been shown to be essential for B cell development, with IL-7-deficient mice having a similar developmental block as the dominant negative Ras transgenic mice (von Freeden-Jeffry et al. 1995). Interestingly, IL-7 and Ras synergize for proliferation of B cell progenitors (Chen et al. 1993; Fleming et al. 2001) and thus inhibition of Ras signaling likely blocks IL-7 dependent proliferation of pro-B cells. Expression of Ras^{N17} after the pro-B cell stage results in defective pre-B cell development and few peripheral B cells showing that Ras activation is required throughout development (Nagaoka et al. 2000). Interestingly, when BCR-Erk signaling was tested in splenic B cells from these mice, there was less than a 50% reduction in Erk activity at early time points. This indicates that the suppression was not complete or that cells in which suppression is not complete are the only ones that survive. Thus, even a reduction in Ras signaling is detrimental to B cell development. Expression of an active form of Ras or a constitutively membrane-bound form of Raf allows for a partial rescue of B cell development in *Rag*$^{-/-}$ mice which lack immunoglobulin heavy or light chain rearrangement indicating that Ras activation is sufficient to mediate some portions of B cell development (Iritani et al. 1997; Shaw et al. 1999).

In *PLC-γ2*$^{-/-}$ mice, there is a deficiency in pro-B cell differentiation resulting in a 30% reduction in peripheral B cells (Wang et al. 2000). Splenic B cell development is also defective due to decreased survival of mature B cells (Bell et al. 2004). Interestingly, only a minor B cell development defect results from the complete lack of BCR-Ras-Erk signaling in *PLC-γ2*$^{-/-}$ mice. This suggests that the more dramatic deficiency seen in Ras^{N17} transgenic mice reflects an inhibition of both BCR- and IL-7-mediated signals.

1.7.4 Ras-Erk activation downstream of other receptors in B cells

A number of receptors are expressed in B cells that act in conjunction with BCR stimulation to generate effector responses. Several of these receptors activate Ras-Erk signaling or interact with this signaling pathway.

CD40 is a 50 kDa transmembrane glycoprotein of the tumor necrosis factor receptor (TNF-R) family expressed on a variety of cells including B cells. The ligand for CD40 is CD154 which is expressed on activated T cells (Armitage et al. 1992; Hollenbaugh et al. 1992). Ligation of CD40 on B cells results in proliferation, upregulation of both adhesion and T cell co-stimulatory proteins, chemokine production, and immunoglobulin class-switching (Bishop et al. 2003). While CD40 ligation on immature B cells results in weak and transient Erk activation (maximal at one minute), mature B cells display robust CD40-Erk signaling (Gulbins et al. 1996; Kashiwada et al. 1996; Li et al. 1996; Sutherland et al. 1996; Purkerson et al. 1998). CD40 ligation results in the association of TRAF (TNF-R associated factors) family members that can then associate with germinal center kinase (GCK). This kinase then activates Mekk1 which in turn activates various Mapks (Yuasa et al. 1998). There is also evidence for other kinases upstream of the Mapks associating with TRAFs in non-B cells and this might also occur upon CD40 ligation (Bishop et al. 2003). Lyn, PI3K, and PLC-γ2 have also been shown to be activated following CD40 ligation in the Daudi human B cell line (Ren et al. 1994), and thus Ras-Erk activation could occur in a similar manner to BCR signaling. However, unlike BCR induced Erk activation, CD40-Erk signaling cannot be inhibited by overnight treatment with PMA in murine splenic B cells (Li et al. 1996), while in Daudi B cells PKC inhibitors could block CD40-Ras signaling (Gulbins et al. 1996). This suggests that at least in some B cells, PLC-γ2-PKC-mediated Ras-Erk activation is not the predominant mechanism. Nonetheless, B cells from Lyn-, PI3K p85-, and PLC-γ2-deficient mice have reduced CD40-mediated responses, demonstrating that this pathway is important *in vivo* (Wang et al. 1996; Fruman et al. 1999; Wang et al. 2000). The activation of Erk following CD40 ligation in B cells is mediated by both Ras-dependent and Ras-independent mechanisms that are likely regulated by the maturation and activation state of the B cell.

Interleukin-4 (IL-4) is a cytokine produced by a subset of T cells (Th2 CD4$^+$, γδ T cells, NKT cells), basophils, mast cells, and eosinophils (Nelms et al. 1999). In B cells, this cytokine promotes survival, immunoglobulin class-switching and is a co-mitogen. B cells express IL-4Rα, which binds to IL-4 with high affinity and allows for common γ chain (γc) association. This brings the associated Janis kinases (JAKs) into close proximity (JAK3 on the γc and JAK1 on the much larger IL-4Rα), resulting in the phosphorylation of the JAKs and the IL-4Rα. STAT6 is then recruited to the phosphorylated IL-4Rα by means of its SH2 domain. This recruitment allows STAT6 phosphorylation by JAKs causing it to dissociate from the receptor, homodimerize via SH2-phosphotyrosine interactions with another phosphorylated STAT6, and then translocate to the nucleus. STAT6 then activates the transcription of a number of genes in co-ordination with other transcription factors such as AP-1. There is evidence for a Grb2-Sos-Ras-Erk pathway downstream of the IL-4R in certain cells, however, this does not appear to be the case in B cells (Nelms et al. 1999). In B cells, the Ras-Erk pathway activated by non-IL-4 signaling synergizes with STAT6 signaling to induce the transcription of a number of genes including the germline ε promoter (for IgE class-switching) and cyclin D2 (Solvason et al. 2000; Shen et al. 2001; Piatelli et al. 2002).

Another effect of CD40 and IL-4 signaling is to alter subsequent signaling from the BCR. Following 2 days of CD40-ligation primary murine B cells have enhanced BCR-Erk signaling, with this enhancement being insensitive to PLC-γ2 inhibitors (Mizuno et al. 2005). IL-4 treatment for a day generates a PLC-γ2-independent BCR-Erk signaling pathway that requires STAT6-mediated protein synthesis (Guo et al. 2005). Thus, IL-4 and CD40 signaling can generate alternate pathways for Erk activation upon BCR-ligation that are not PLC-γ2-mediated.

1.7.5 Ras-mediated B cell responses

In response to an antigen encounter, B cells can activate, proliferate and differentiate. The ultimate goal of a B cell is to secrete antigen-specific antibody in order to clear the pathogen. B cells that have fully developed are ready to mediate effector responses. However, given the large number of different BCR specificities, very few B cells can recognize a given pathogen. One mechanism to combat the small number of pathogen-specific B cells is antigen-specific proliferation. Ligation of the BCR results in proliferation of B cells that is abrogated by inhibitors of Mek (Richards et al. 2001). Similarly, PLC-γ2-deficient B cells, which lack BCR-Erk signaling, have defective proliferation upon BCR stimulation (Wang et al. 2000; Bell et al. 2004). IL-4 increases B cell survival and is also capable of substantially increasing the proliferation induced by BCR ligation (Nelms et al. 1999). While IL-4-mediated enhanced B cell survival is unaffected by Mek inhibition, proliferation is substantially reduced in the absence of Erk activation (Richards et al. 2001). Also, expression of a constitutively active Ras is sufficient to synergize with IL-4 signaling to induce B cell proliferation (Levings et al. 1999). These results indicate that Ras-Erk signaling is the primary pathway mediating BCR-induced synergy with IL-4 to induce proliferation. CD40 and BCR signaling are also synergistic in terms of proliferation despite only having an additive effect on early Erk activation (Li et al. 1996). B cells stimulated with CD40 ligation alone are induced to proliferate. However, Fas expression is upregulated in this case and signaling from another receptor such as IL-4R or BCR is required to save these cells from being killed by activated T cells expressing FasL (Foote et al. 1996; Foote et al. 1998; Hinshaw et al. 2003).

While recognition of antigen by the BCR is the first step in the B cell immune response, secretion of antibody is the primary mechanism by which pathogens will be cleared. B cells must differentiate into antibody secreting cells and many of them class-switch from IgM/IgD to one of the other immunoglobulin isotypes depending on the contextual signaling cues and the type of antigen recognized. B cell division is required for immunoglobulin isotype class-switching and the generation of antibody secreting B cells which are characterized by the expression of CD138 (Hodgkin et al. 1996; Abdel-Latif et al. 2005). As such, processes which affect B cell proliferation will not only affect the number of B cells but also the proportion of these cells which differentiate and activate (Tangye et al. 2004). There is also evidence of Erk signaling having a direct effect on class switching due to the presence of AP-1 binding sites in the promoters of germline heavy chain transcripts (Grant et al. 1995; Shen et al. 2001). Transcription of these regions without translation is required for class switching (Snapper et al. 1997; Zhang 2003).

There are two general categories of antigens capable of inducing a B cell-mediated immune response, those that are T cell-dependent (TD) or T cell-independent (TI). TD antigens are usually proteins that upon BCR binding are internalized and digested into peptide fragments that are presented on the surface in complex with MCH class II molecules. If a T cell recognizes one of these peptides it will give activation signals to the B cell allowing it to proliferate, activate and differentiate (Parker 1993). TI antigens can be divided into type 1 (TI-I) and type 2 (TI-II) (Mond et al. 1995; Vos et al. 2000). TI-I antigens are polyclonal B cell activators which means that in addition to potentially ligating the BCR they are recognized by non-polymorphic receptors on B cells and thus cause proliferation independent of T cells. Lipopolysaccharide, a bacterial cell wall component, is a well-characterized TI-I antigen. TI-II antigens are highly repetitive molecules that cross-link the BCR extensively. These antigens are not as potent B cell activators as TI-I immunogens and additional stimulatory signals are required to induce a robust immune response.

The role of BCR signaling proteins in the immune response are difficult to assess using knockout mice with B cell development defects. *PLC-$\gamma 2^{-/-}$* mice have approximately 2/3 the normal number of splenic B cells due to partial developmental blocks in the BM and spleen (Wang et al. 2000; Bell et al. 2004). In these mice the TI-II response is completely lacking while the TD response is altered but not absent (Wang et al. 2000). This indicates that T cell contact-mediated signals are sufficient to rescue partially the defects seen in these mice. PI3K p110δ knockout mice, which have defective B cell proliferative responses, have deficient TI-I responses while TI-II and TD responses are completely defective (Clayton et al. 2002; Jou et al. 2002). Similarly, Vav1/2/3 triple knockout mice lack B cell proliferative responses and immune responses (Fujikawa et al. 2003). Thus, mice that are defective in BCR signaling often display reduced B cell proliferation and antigen-specific immune responses.

1.8 Hypothesis

RasGRP1 and RasGRP3 both activate Ras and possesses functional DAG-binding C1 domains. T cells express RasGRP1, which links TCR signals to Ras activation. Since RasGRP1 and RasGRP3 are both expressed in B cell lines and TCR signaling shares many similarities with BCR signaling, I hypothesized that RasGRP1 and/or RasGRP3 connect BCR signaling to Ras in murine B cells. Ras-Erk signaling is essential during B cell development as well as during B cell immune responses. Therefore, I also proposed that mice deficient in BCR signaling to Ras would show substantial B cell defects at the level of B cell development and proliferation. These two deficiencies would then lead secondarily to diminished B cell activation and effector functions. To test these hypotheses, newly generated *Rasgrp3*$^{-/-}$ mice were mated to *Rasgrp1*$^{-/-}$ mice in order to examine B cell development, BCR signaling and B cell effector functions.

	mRNA	protein
RasGRP1	B cells, T cells, brain, thymus, spleen, kidney, lymph node, keratinocytes	B cells (splenic), T cells (DP and SP thymocytes, splenic and lymph node T cells), brain, keratinocytes
RasGRP2	Brain, heart, kidney, placenta, lung, liver, skeletal muscle, pancreas, spleen, megakaryocytes, bone marrow, lymph node, thymus, T cell, B cell, myeloid cells	Brain, megakaryocytes and platelets, neutrophils
RasGRP3	Brain, heart, lung, kidney, skeletal muscle, liver, placenta, blood vessels, testis, pancreatic islets	B cells (splenic)
RasGRP4	Peripheral blood leukocytes (myeloid cells), bone marrow, lung	Mast cells

Table 1-1. Tissue expression of RasGRP family members. See text for references.

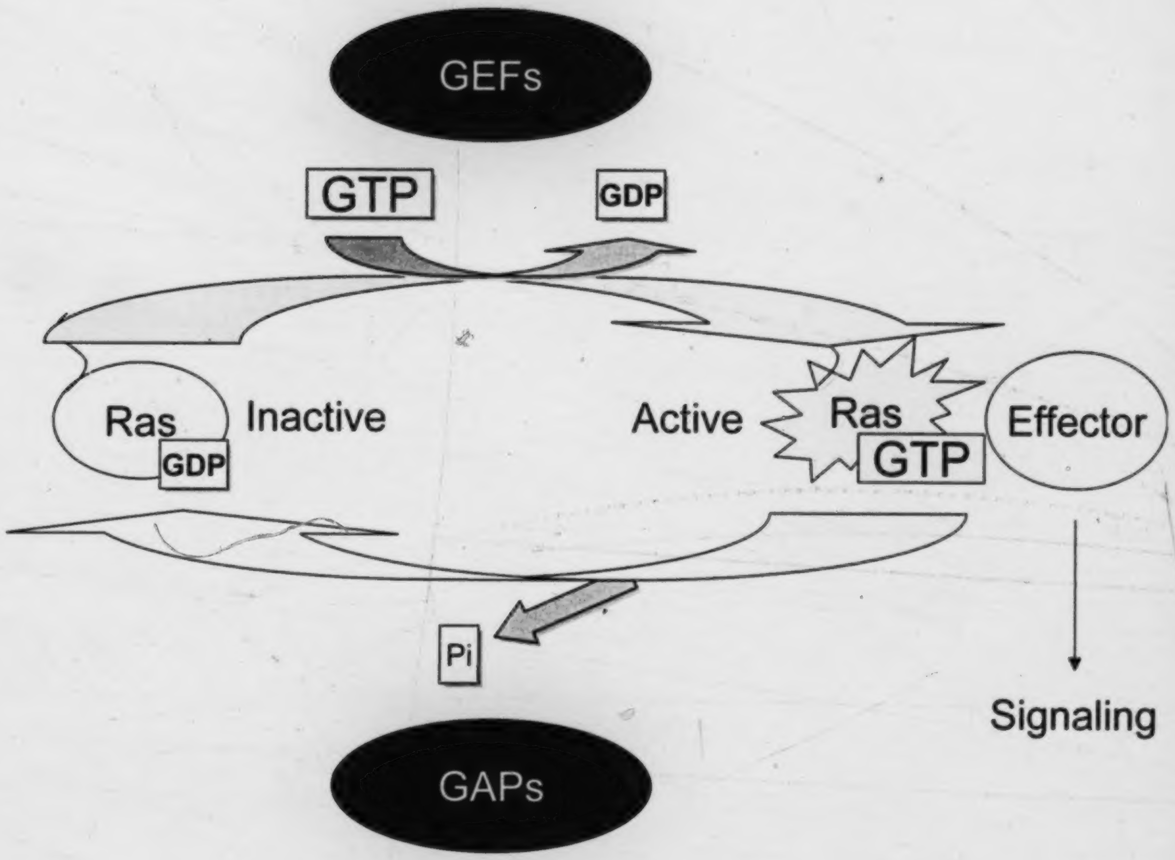
GEFs
GTP
GDP
Ras
GDP
Inactive
Active
Ras
GTP
Effector
Pi
Signaling
GAPs

Figure 1-1. Ras cycle of GDP- and GTP-binding. Membrane-bound Ras cycles between inactive GDP-bound and active GTP-bound states with the assistance of Ras GEFs and Ras GAPs. Ras is activated by the dissociation of GDP which is facilitated by GEFs, and the subsequent binding of the more prevalent GTP. Ras is inactivated by the hydrolysis of GTP which is accelerated by GAPs.

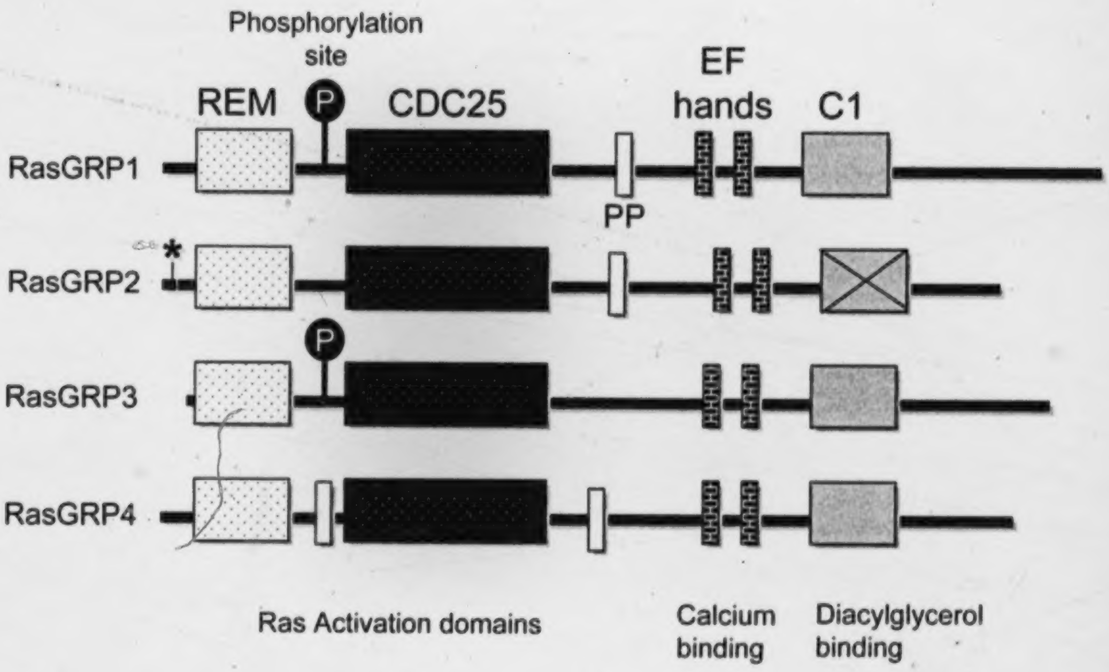
Phosphorylation site
REM
P
CDC25
EF hands
C1
RasGRP1
PP
*
RasGRP2
P
RasGRP3
RasGRP4
Ras Activation domains
Calcium binding
Diacylglycerol binding

Figure 1-2. Schematic diagram of the domain structure of the RasGRPs. The REM (Ras exchange motif) and CDC25 domains are involved in activation of the Ras subfamily of small G proteins. EF-hands are a calcium-binding motif (has only been demonstrated with RasGRP1). C1 domains bind calcium (with the exception of RasGRP2). PP is a polyproline region. Asterisk (*) denotes the myristoylation motif present on certain differentially spliced forms of RasGRP2.

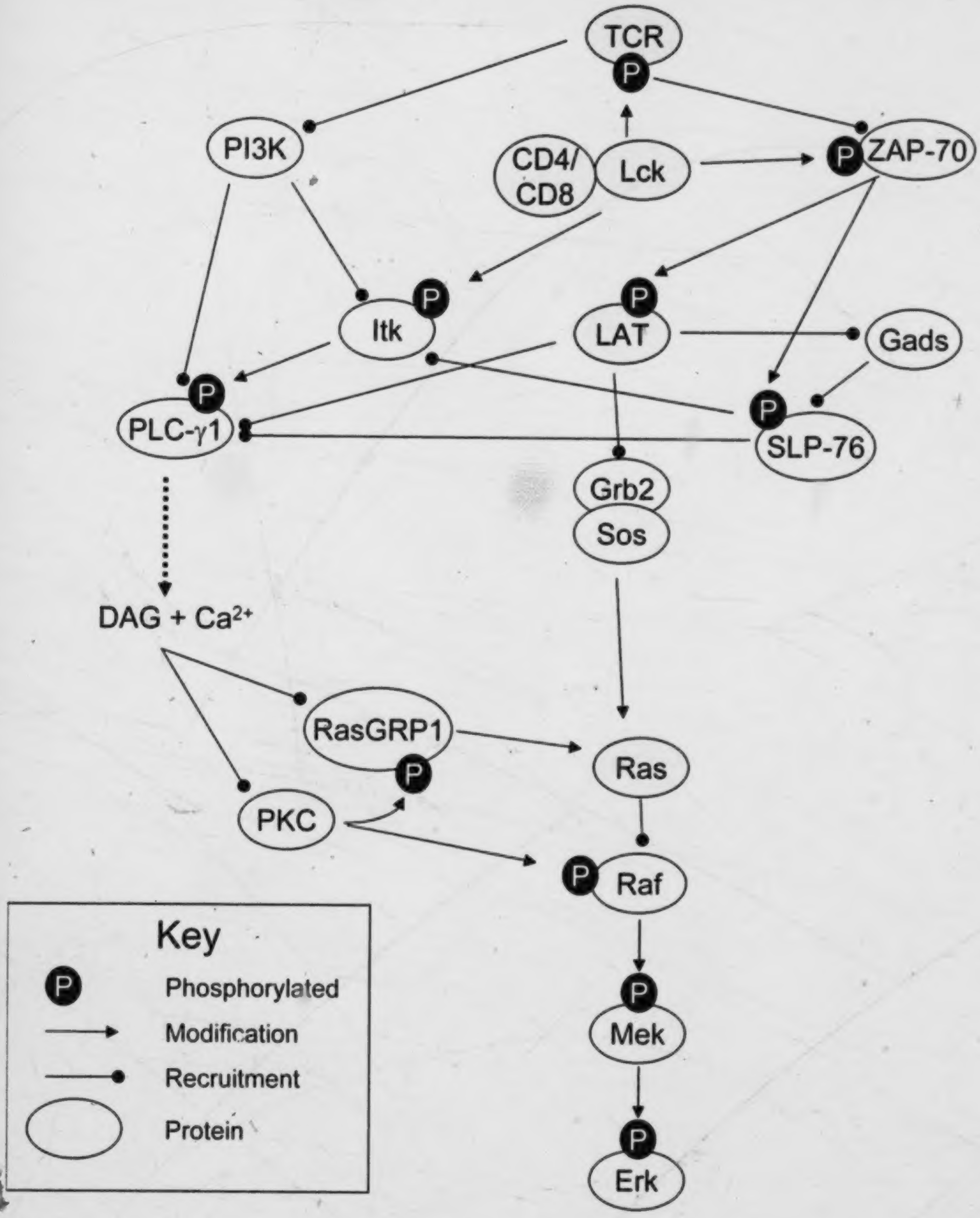
TCR
P
PI3K
CD4/
CD8
Lck
P
ZAP-70
P
Itk
P
LAT
Gads
P
PLC-γ1
P
SLP-76
Grb2
Sos
DAG + Ca2+
RasGRP1
P
Ras
PKC
P
Raf
P
Mek
P
Erk
Key
P
Phosphorylated
Modification
Recruitment
Protein

Figure 1-3. T cell receptor signaling leading to Ras activation. For simplicity, signaling which does not directly lead to Ras activation as well as divergent signaling at the level of Ras have been omitted. Grey background of proteins denotes constitutive membrane association. See key for other representations used and text for details.

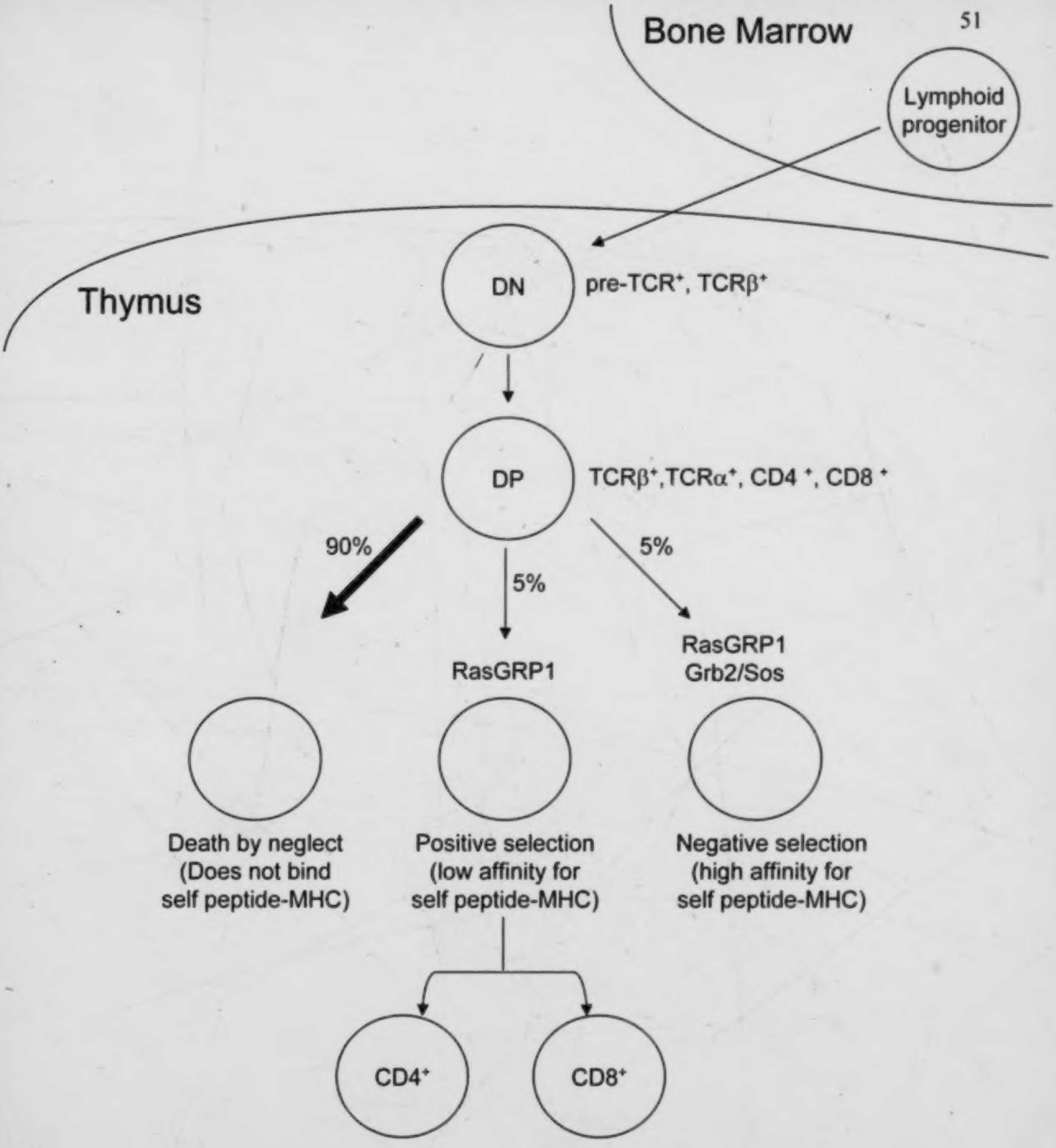
Bone Marrow
Lymphoid progenitor
Thymus
DN
pre-TCR⁺, TCRβ⁺
DP
TCRβ⁺,TCRα⁺, CD4 ⁺, CD8 ⁺
90%
5%
5%
RasGRP1
RasGRP1
Grb2/Sos
Death by neglect (Does not bind self peptide-MHC)
Positive selection (low affinity for self peptide-MHC)
Negative selection (high affinity for self peptide-MHC)
CD4⁺
CD8⁺

Figure 1-4. T cell development. T cell progenitors arrive in the thymus from the bone marrow. Rearrangement of the TCRα and TCRβ loci lead to the expression of a TCR on the surface of a CD4/CD8 DP thymocyte. The majority of T cells express a TCR that does not recognize self peptide-MHC leading to death of these cells by neglect. If the TCR has too high an affinity for self peptide-MHC then both RasGRP1 and Grb2/Sos pathways are activated leading to negative selection. If the TCR has low affinity for self peptide-MHC then only RasGRP1 mediates Ras activation resulting in positive selection and survival of the T cell.

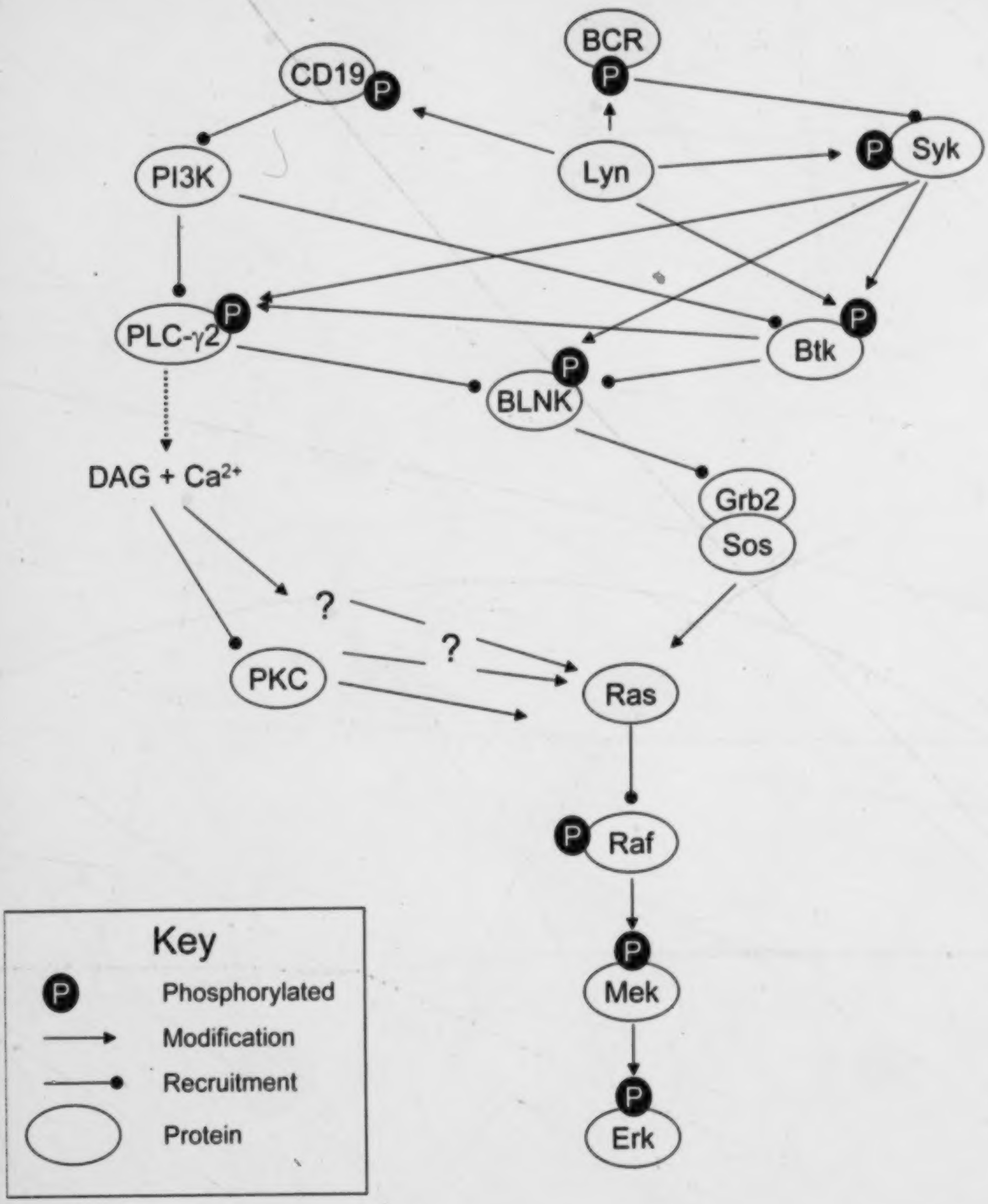
BCR
P
CD19
P
Lyn
P
Syk
PI3K
PLC-γ2
P
P
Btk
P
BLNK
DAG + Ca2+
Grb2
Sos
?
?
PKC
Ras
P
Raf
P
Mek
P
Erk
Key
P
Phosphorylated
Modification
Recruitment
Protein

Figure 1-5. BCR-induced signaling leading to Ras activation. For simplicity, signaling which does not directly lead to Ras activation as well as divergent signaling at the level of Ras have been omitted. Grey background of proteins denotes constitutive membrane association. See key for other representations used and text for details.

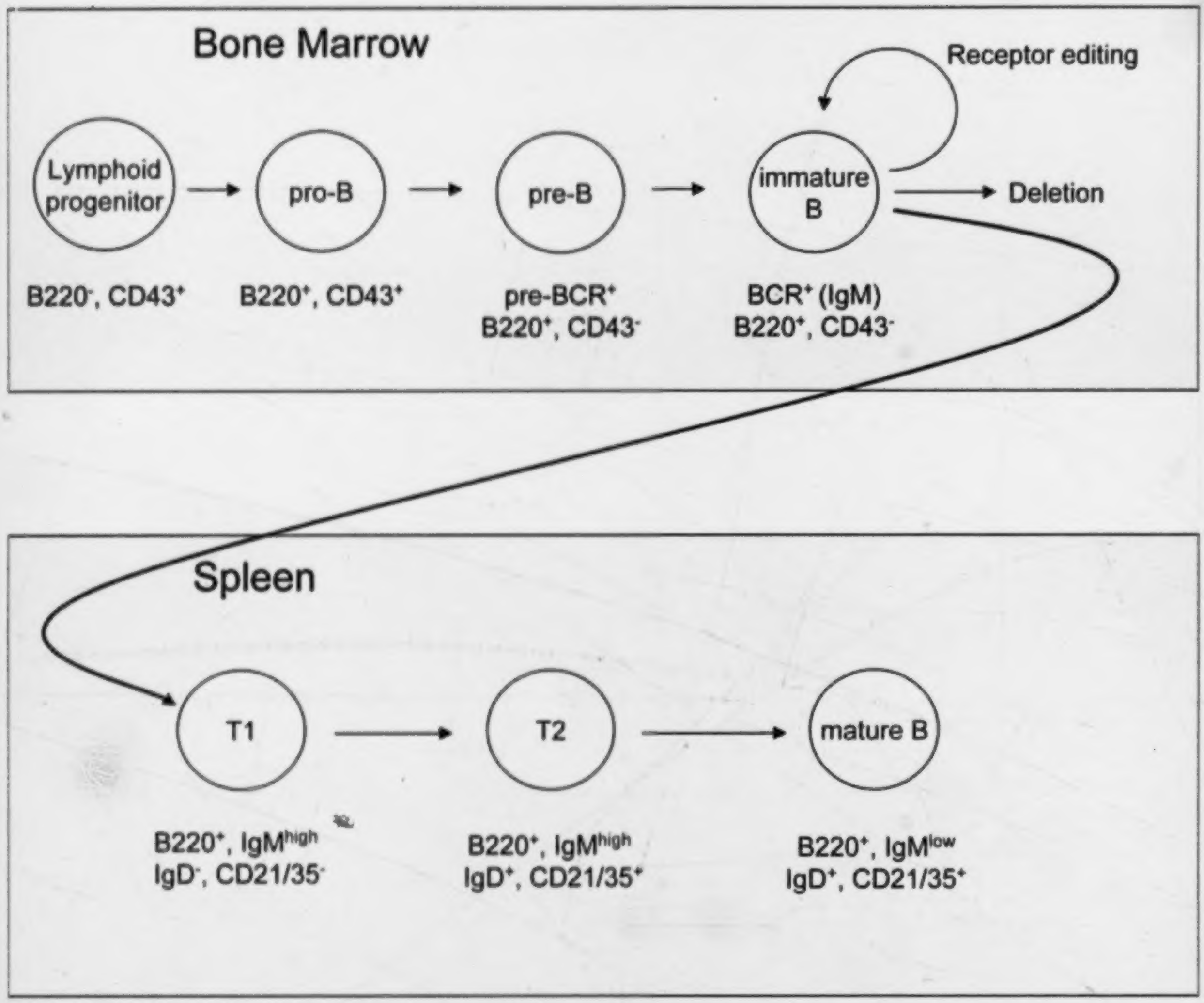
Bone Marrow
Receptor editing
Lymphoid progenitor
pro-B
pre-B
immature B
Deletion
B220$^-$, CD43$^+$
B220$^+$, CD43$^+$
pre-BCR$^+$ B220$^+$, CD43$^-$
BCR$^+$ (IgM) B220$^+$, CD43$^-$
Spleen
T1
T2
mature B
B220$^+$, IgM^{high} IgD$^-$, CD21/35$^-$
B220$^+$, IgM^{high} IgD$^+$, CD21/35$^+$
B220$^+$, IgM^{low} IgD$^+$, CD21/35$^+$

Figure 1-6. B cell development. B cell progenitors in the bone marrow rearrange their heavy chain genes. The protein is then expressed on the surface along with the surrogate-light chain to form the pre-BCR. After several cell divisions the light chain gene rearranges and associates with the heavy chain to form the BCR. If the BCR is self-reactive, the immunoglobulin is either receptor-edited (to alter the specificity) or the B cell is deleted. B cells expressing a functionally rearranged and non-self reactive BCR travel to the spleen to continue development. In the spleen Transitional 1 (T1) and T2 B cells are still subject to negative selection. T2 B cells represent the first stage at which BCR ligation results in proliferation rather than deletion although it is not until the mature B cell stage that B cells are fully immunocompetent.

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Chapter 2

Materials and Methods

Chapter

2

Material and Methods

2.1 Mice

Rasgrp3 mutant mice were derived on a 129/J and C57BL/6J (B6) hybrid background by others in the lab in collaboration with Dr. Peter Dickie essentially as described previously for *Rasgrp1* mutant mice (Dower et al. 2000). Briefly, a *neo* cassette was inserted into the BstX1 site in the second exon of *Rasgrp3* interrupting the coding sequence downstream of codon 40 in Ras Exchange Motif (REM box) of the catalytic region. *Rasgrp3* null mice were viable, fertile and lacked overt phenotype (as determined by those listed above). It was previously reported that homozygous *Rasgrp1* null mice displayed reduced viability in this background (Dower et al. 2000), but such an effect was not evident in my colony. In crosses between *Rasgrp1*$^{-/-}$ and *Rasgrp3*$^{-/-}$ mice on a mixed genetic background, single and double homozygous mice were observed in approximately expected Mendelian ratios, as these genes are located on different chromosomes. Mutant mice derived on the 129/J genetic background were mated to C57BL/6J mice to enhance fertility. The mice used in this study are from 129/J; C57BL/6J F1 crosses between mice whose genotype is *Rasgrp1*$^{+/-}$;*Rasgrp3*$^{+/+}$ thereby generating WT and 1KO mice as well as crosses between mice whose genotype is *Rasgrp1*$^{+/-}$;*Rasgrp3*$^{-/-}$ thereby generating 3KO and DKO mice.

The genotype of all homozygous null mutant mice was determined by PCR as established by others in the lab (Figure 2-1 and 2-2). Briefly, each mouse tail was digested overnight at 55°C in tail buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.455 Tween 20, protease K 0.5 mg/ml). Samples were heat-inactivated at 94°C for 10 minutes and then 2.5 ul was used for 25 ul PCR reactions using the primer combinations listed in Figures 2-1 and 2-2.

Genotyping results were later verified by immunoblotting protein extracts with RasGRP1 and RasGRP3 antibodies generated by others in the lab (see section 2.2). Nude mice (C57BL/6J genetic background) were purchased from The Jackson Laboratory and genotyped as per supplier's protocol. Mice were bred under VAF conditions. All animal studies were done according to protocols approved by The Health Sciences Animal Policy and Welfare Committee at the University of Alberta, in accordance with Canadian Council on Animal Care Guidelines.

2.2 Reagents and antibodies

Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-mouse anti-IgM was from SouthernBiotech (Birmingham, AL), hamster anti-mouse CD40 (HM40-3) was from BD Biosciences (San Jose, CA) recombinant mouse interleukin-4 (IL-4) was from Biosource (Camarillo, CA), anti-mouse IL-4 (11B11) was from eBiosciences (san Diego, CA) anti-phosphotyrosine (4G10) and pan-Ras antibody (RAS10) were from Upstate Biotechnology (Lake Placid, NY), phospho-Erk (9101), STAT6 and phospho-STAT6 antibodies were from Cell Signaling Technology (Beverly, MA), alexa488 conjugated goat anti-mouse Ig and 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) were from Molecular Probes (Eugene, OR), Rap-1(SC-65) and cyclin D2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), SDF-1α was from R and D systems (Minneapolis, MN), and the hybridoma for anti-CD3ε (145-2C11) was a gift from Dr. Hanne Ostergaard (University of Alberta). Antibodies directed against RasGRP1 (Puente et al. 2000) and RasGRP3 (Teixeira et al. 2003) have been previously described. RasGRP2 and RasGRP4 were not studied because the former acts on Rap and does not bind DAG while the latter is expressed in non-T, non-B cell types. 2,4,6, trinitrophenyl (TNP)-Bovine Serum Albumin (BSA), TNP-LPS, TNP-Keyhole Limpet Hemocyanin (KLH), dinitrophenyl (DNP)-Ficoll and DNP-BSA were purchased from Biosearch Technologies (Novato, CA).

2.3 Cell enrichment and immunofluorescence

All experiments presented are representative of at least three independent experiments that yielded similar results. B cells and total or $CD4^+$ T cells were enriched to greater than 95%, as determined by flow cytometry, by negative selection of undesired splenocytes, using B cell, pan T cell or $CD4^+$ T cell isolation kits respectively as per manufacturer's instructions (Miltenyi Biotec, Auburn, CA). B cell isolation kits remove cells expressing CD43 (several non-B cell types, activated B cells, plasma B cells, B1 cells), CD4 (T cell subset), and Ter-119 (erythrocytes). B cells isolated by this method were either IgM^+ or IgM^{low} and all were lacking CD69 plus CD138 expression. Alternatively, treatment of splenocytes with 20 ug/ml LPS for 3 days resulted in enrichment of $B220^+$ cells to greater than 85%. Anti-nuclear antibodies (ANA) were detected on HEp-2 slides (BIO-RAD, Redmond, WA) using serum samples from 3-6 month old mice at a 1:50 dilution as per protocol obtained from Keith Elkon. HEp-2 slides were pre-wet with PBS for 5 minutes followed by 30 minutes incubation at room temperature with 60 ul of 1:25 and 1:50 dilutions of each sample. Following washing, Alexa488 conjugated goat anti-mouse Ig was added at a 1:50 dilution for 30 minutes at room temperature. After the slides were washed, digital photomicrographs were taken using a Zeiss Axioskop II microscope and a Diagnostic Instruments SPOT 1.3.0 charge-coupled device digital camera.

2.4 Signaling assays and quantitation of RasGRP1 plus RasGRP3 expression

For signaling experiments, splenic B cells from 5-6 week old mice were incubated at 37°C for 30 minutes in serum-free RPMI after enrichment at 0-4°C. Equal numbers of cells were then treated for indicated times with either 100 nM PMA or 20 ug/ml anti-IgM for Ras and Erk activation, or with various combinations of IL-4 (0-10 ng/ml) and anti-IgM (1 ug/ml) for cyclin D2 induction and phospho-STAT6 signaling. Cells were then recovered by centrifugation and lysed as previously described (Teixeira et al. 2003). The Ras activation assay has been previously described (Teixeira et al. 2003). The Rap activation assay was performed in the same manner to the Ras activation assay with RalGDS-GST (a gift from Dr. Mike Gold) replacing Raf(RBD)-GST. Lysates were run on SDS-PAGE, transferred to a filter and probed with the indicated antibodies. For quantitation of RasGRP1 and RasGRP3 expression, the concentrations of maltose-binding protein (MBP, approximately 42 kDa)-tagged forms of these proteins (produced by Yong Zheng) were visually approximated by SDS-PAGE followed by coomasie blue staining along with a BSA standard. Dilutions of the recombinant proteins along with B cell lysates were analyzed by western blot with anti-RasGRP1 followed by anti-RasGRP3.

2.5 Flow cytometry

Single-cell suspensions of live splenocytes were stained with various fluorescently labeled antibodies in buffer (PBS, 0.5% BSA) for 30 minutes on ice, and then washed 2 times. Most antibodies were used at a 1:50 dilution with the exception of anti-IgM that was used at 1:10 and anti-B220 that was used at 1:400. Antibodies to CD19 (1D3), IgG1 (X56), B220 (RA3-6B2), CD8 (53-6.7), CD138 (281-2), CD69

H1.2F3), CD4 (L3T4), IgD (11-26c.2a), CD5 (53-7.3), and CD43 (57) along with propidium iodide were purchased from Becton Dickinson (San Jose, CA). Following cell staining 10 ul of propidium iodide solution was added, cells were mixed, and then analyzed by flow cytometry using a Becton Dickinson FACScan, FACSCalibur or FACSCanto and analyzed using Flowjo software (Tree Star, Ashland, OR). Viability of cells was determined by cell scatter properties and propidium iodide exclusion. Determination of IgG1 expression has been described (Hasbold et al. 2004). Briefly, cells were fixed with 2% paraformaldehyde and then permeabilized overnight with 0.1% Tween-20. Cells were then washed and stained according to standard procedures.

2.6 Immunizations and ELISA

To determine immune responses, mice were immunized by intraperitoneal injection with 100 ug TNP-KLH (T-dependent), 20 ug TNP-LPS (T-independent type I) or 100 ug DNP-Ficoll (T-independent type II) in 100 ul PBS on days 0 and 14. Mice were bled before the first immunization (day 0) as well as on days 7, 14 (before secondary immunization), 21 and 28. For aged basal serum antibody titers, 3-6 month old mice were bled. Antibody isotypes were quantified using SBA Clonotyping System/HRP (SouthernBiotech, Birmingham, AL) following the manufacturer's instructions. For total antibody levels, plates were coated with goat anti-mouse Ig capture antibody, while for TNP- or DNP-specific antibody levels plates were coated with 10 ug/ml TNP- or DNP-BSA respectively. Values were obtained by serially diluting samples and using dilutions that fell within a standard curve using isotype specific controls (SouthernBiotech, Birmingham, AL).

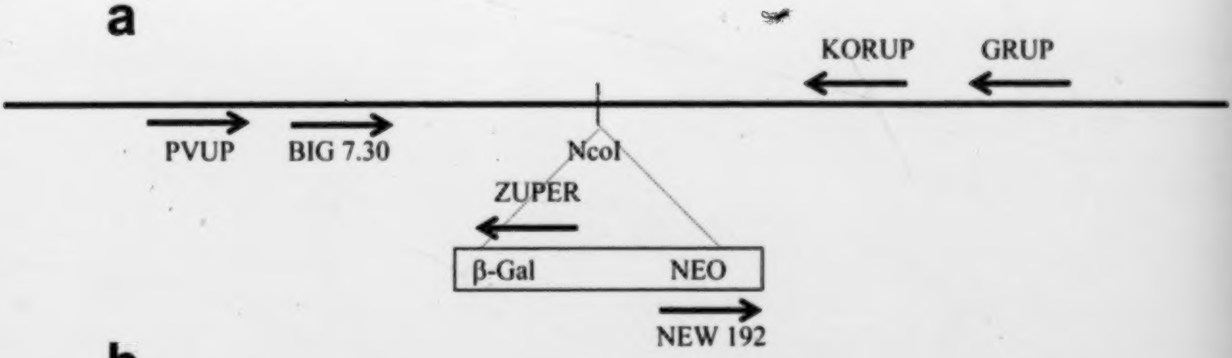
2.7 Proliferation and activation assays

For CFSE-labeling, B cells and CD4$^+$ T cells were isolated from the spleens of 6 week old mice and labeled according to the originally published method (Lyons et al. 1994). B cells labeled by this technique cannot be analyzed for two days due to the breakdown of short-lived proteins that have been labeled. B cells were cultured at 10^5 cells in 1 ml of media (RPMI supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and antibiotics) with various combinations of the following: 20 ug/ml anti-IgM, 0.1 or 10 ng/ml IL-4, 10 ug/ml anti-CD40, 20 ug/ml LPS. Following 4 day treatments, cells were analyzed by flow cytometry. For mixing experiments, RasGRP1-deficient CD4$^+$ T cells were obtained from double knockout mice to ensure the onset of autoimmunity would not affect the results. RasGRP3-deficient mice were used as a source of wildtype T cells. Similarly, CD4$^+$ T cells were used rather than total T cells in order to obtain similar populations since *Rasgrp1*$^{-/-}$ mice have few CD8$^+$ T cells. CD4$^+$ T cells were cultured at 10^6 cells in 1 ml of media on anti-CD3ε coated plates (10 ug/ml for 1 hour at room temperature) along with 10^5 B cells and anti-IgM. Four days later, cell mixtures were fluorescently labeled with anti-B220 plus anti-CD19 and were analyzed by flow cytometry. In control experiments analysis of expression of these two surface markers allowed for differentiation of T cell and B cell populations after four days of stimulation. B cells were also treated with conditioned media from cultured T cells stimulated for 8 days with anti-CD3ε. In this case, supernatants were mixed 1:1 with fresh media with or without 1 ug/ml anti-IL-4 (11B11) for one hour and then CFSE-labeled B cells were added at 10^5 cells in 1 ml. Again, cells were analyzed by flow cytometry 4 days later. For all CFSE-labeling experiments, equal numbers of cells were analyzed although flow cytometry histograms are shown with different axis scales because of the reduced viability of double knockout B cells. Average number of cell divisions and percent of cells divided were calculated by analyzing flow cytometry results using the Flowjo proliferation platform.

Alternatively, bromodeoxyuridine (BrdU) labeling was used to detect S phase cells. Splenic B cells were isolated and then 10^6 cells/ml were allowed to proliferate in media alone or in combination with indicated stimulus. After three days incubation, cells were exposed for 1 hour to BrdU in culture medium. Cells were then fixed, permeabilized and stained with anti-BrdU and 7-amino-actinomycin D (7-AAD) following the manufacture's instructions (Becton Dickinson, San Jose, CA). Cells were analyzed by flow cytometry. Due to the flow cytometer settings used, differentiation between dead cells and debris could not be performed; dead cells and debris were both gated out based on forward and side scatter properties.

2.8 Migration assay

Isolated B cells (10^6) were placed into the upper chamber of transwell inserts in 100 ul of migration media. The lower chamber contained 600 ul of migration media (RPMI plus 0.5% BSA) with or without SDF-1 (100 ng/ml). Cells were left to migrate for 3 hours at 37°C and then those cells that had migrated into the lower chamber were counted in triplicate using a coulter counter. Transwell plates (#3421, polycarbonate filters with a pore size of 5 um and thickness of 10 um) were from Corning Inc. (Corning, NY).



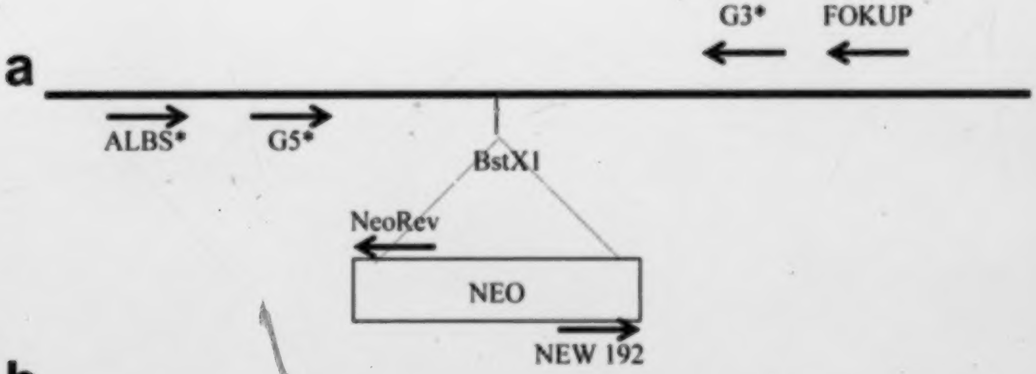
b

BIG 7.30	5' CTA TCC TCA CTT GAG TCT CTC TTT CC 3'
PVUP	5' CTG CCA TTG AGA CCC CCA GCT GC 3'
NEW 192	5' ATA TTG CTG AAG AGC TTG GCG GCG AAT GGG 3'
ZUPER	5' CCG TAA TGG GAT AGG TTA CGT TGG TG 3'
GRUP	5' GCC GTA AGC GCT ACA GAC TGT CTC C 3'
KORUP	5' GCA GCT GCT AAT AAG ATC ATC CAG GC 3'

c

		GENOTYPE:		
Primer combination	Expected size	+/+	+/-	-/-
BIG 7.30/KORUP	202 bp	+	+	-
PVUP/GRUP	396 bp	+	+	-
NEW 192/KORUP	317 bp	-	+	+
BIG 7.30/ZUPER	431 bp	-	+	+

Figure 2-1. ***Ragrp1*** **PCR genotyping.**
(a) Schematic diagram of the insertion made in *Rasgrp1* with the relative positions and orientations of the primers indicated. **(b)** Primers used for PCR typing. **(c)** The primer combinations used for genotyping along with the size of the resulting product (bp, base pairs). The genotype of the mouse can be determined by the presence or absence of these PCR products as listed which indicate the presence of a wildtype (+) or a gene targeted (-) allele.



b

	5' 3'
NeoRev	CGA GCA CAG CTG CGC AAG GAA CGC
	5' 3'
G5*	GAT GAC AAT GGA GAC CTG AAC GAC AG
	5' 3'
ALBS*	TAG CTG TCC CTT GTG AGA CTA GGC CG
	5' 3'
NEW 192	ATA TTG CTG AAG AGC TTG GCG GCG AAT GGG
	5' 3'
FOKUP	CGA CTT GGC CCA TCA TCC AGA TGT C
	5' 3'
G3*	CAT CTA TAT AAC CTC AAA ACA GCT GG

c

		GENOTYPE:		
Primer combination	Expected size	+/+	+/-	-/-
G5*/NeoRev	530 bp	-	+	+
NEW192/FOKUP	310 bp	-	+	+
ALBS/FOKUP	470 bp	+	+	-
G3*/ALBS	435 bp	+	+	-

Figure 2-2. ***Ragrp3*** **PCR genotyping.**
(a) Schematic diagram of the insertion made in *Rasgrp3* with the relative positions and orientations of the primers indicated. **(b)** Primers used for PCR typing. **(c)** The primer combinations used for genotyping along with the size of the resulting product (bp, base pairs). The genotype of the mouse can be determined by the presence or absence of these PCR products as listed which indicate the presence of a wildtype (+) or a gene targeted (-) allele.

2.10 References

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Chapter 3
Results

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Chapter

3

Results

3. 1 RasGRP1 and RasGRP3 link BCR signaling to Ras

In order to evaluate the role of RasGRP1 and RasGRP3 in B cells, I first determined if both proteins are expressed in murine splenic B cells as they are in several B cell lines (Oh-hora et al. 2003; Teixeira et al. 2003; Ehrhardt et al. 2004; Guilbault et al. 2004). As expected, wildtype B cells isolated from the spleen express both RasGRP1 and RasGRP3 while T cells express only RasGRP1 (Figure 3-1a). These proteins are missing in lysates from the corresponding knockout mice, which others in the lab generated (see section 2.1). Interestingly, T cells express far more RasGRP1 than do B cells and the RasGRP3 expression is substantially greater than RasGRP1 in B cells (Figure 3-1b). In addition, the expression level of one RasGRP species is not affected by the expression status of the other.

Splenic B cells from wildtype and mutant mice were used to evaluate the contribution of RasGRP1 and RasGRP3 to Ras-Erk signaling. Treatment of wildtype B cells with the DAG analogue PMA resulted in rapid accumulation of Ras-GTP and phosphorylated Erk (Figure 3-2). Splenic B cells from either *Rasgrp1*$^{-/-}$ or *Rasgrp3*$^{-/-}$ mice also exhibited activation of Ras-Erk signaling in response to PMA. In contrast, *Rasgrp1;Rasgrp3* double knockout B cells displayed no Ras activation and only weak

Erk phosphorylation in response to PMA stimulation indicating that both RasGRP1 and RasGRP3 are DAG-responsive Ras activators in B cells.

Next, I assessed the effect of BCR ligation on Ras activation in wildtype and mutant mice using anti-IgM antibodies. Compared to PMA treatment of cells, activation of Ras and Erk by BCR ligation was weaker and more transient (Figure 3-2a). Relative to wildtype, *Rasgrp1*$^{-/-}$ B cells showed weaker and more transient Ras and Erk activation. Interestingly, *Rasgrp3*$^{-/-}$ B cells exhibited undetectable basal Ras-GTP levels as well as defective BCR-induced Ras activation. At later time points, the activation of Erk was intermediate in each mutant compared to wildtype, and totally absent in the double mutant cells. These results indicate that, while RasGRP1 and RasGRP3 each have a distinct role in regulating Ras in B cells, they also exhibit significant functional redundancy.

Ras activation was also assessed in a longer time course experiment. This was performed in order to determine if there are any other DAG-sensitive Ras activators present in these cells that function at later time points, as has been suggested for RasGRP2 (see section 1.4.2). In order to obtain sufficient numbers of cells, I expanded splenocytes in culture with LPS to enrich B cells and assessed Ras activation following PMA stimulation (Figure 3-2b). PMA induced Ras activation in wildtype B cells for the entire duration of the assay (5 minutes to 24 hours) although Erk phosphorylation began to decrease by 4 hours. Similar results were observed in each of the single mutant mice although Ras and Erk activation were absent in *Rasgrp3*$^{-/-}$ B cells at 24 hours. However, PMA failed to induce Ras activation in double knockout B cells during this time course and there was only weak Erk phosphorylation. These results further confirm that RasGRP1 and RasGRP3 are the only DAG-responsive Ras activators in B cells. Interestingly, PMA treatment resulted in decreased RasGRP1 expression while the expression of RasGRP3 was not decreased (Figure 3-2c). This is likely the reason for the lack of Ras-GTP at 24 hours in *Rasgrp3*$^{-/-}$ B cells. A shift in RasGRP3 mobility was also observed upon PMA treatment which has been previously shown to correlate with phosphorylation (Teixeira et al. 2003; Zheng et al. 2005).

I used flow cytometry to assess the surface expression of various proteins on the isolated B cells in order to ensure that the defective Ras activation in double knockout B cells was not a result of an altered starting population. The B cells isolated were of high purity (>95% B220$^+$) and expressed similar levels of IgM on their surface (Figure 3-3a). There were very few B cells expressing CD138 or CD69 which are markers of plasma cells and activation respectively (Figure 3-3b and c). These results suggest that the isolated B cells from each genotype were similar in developmental and activation status. To ensure that the defective Ras activation in double knockout B cells was not a result of globally defective BCR signaling, I examined total tyrosine phosphorylation following anti-IgM treatment (Figure 3-4). Numerous phosphorylated proteins were observed upon BCR stimulation, and this pattern was not significantly affected by genotype of the B cells. The flow cytometry and tyrosine phosphorylation results argue that the defects in Ras and Erk activation in double knockout mice are specific, direct effects of reduced Ras GEF activity. Furthermore, the equivalent cell populations and phospho-tyrosine responses demonstrate that the isolated B cells from the four different genotypes are functionally equivalent in several respects.

3.2 RasGRP1 and RasGRP3 are not required for Rap activation

BCR-ligation and PMA can both induce the activation of Rap, a Ras superfamily small G protein, in B cells (McLeod et al. 1998). Given that RasGRP3 can activate Rap, I assessed whether RasGRP3 mediates Rap activation in B cells. Using LPS-expanded B cells, I analyzed the activation of Rap. PMA was able to induce approximately equivalent fold Rap activation in B cells from mice of all four genotypes (Figure 3-5a).

I next determined whether Rap-mediated B cell migration was intact. Stromal cell-derived factor-1 (SDF-1) is a chemokine that induces B cell migration. SDF-1 binding to its receptor, CXCR4, induces the activation of PI3K, PLC-γ2, Rap, Mek, and Erk (Ganju et al. 1998; Tilton et al. 2000; Wang et al. 2000; McLeod et al. 2002). SDF-1 signaling to Rap activation is blocked by the addition of PLC inhibitors and inhibition of Rap activation by means of RapGAP expression greatly reduces SDF-1-induced migration (McLeod et al. 2002). The PLC-dependent Rap activation combined with Erk activation suggests a possible role for RasGRP3, which is a dual specific Ras/Rap GEF. RasGRP1-deficiency had a mild inhibitory effect on SDF-1 induced migration while RasGRP3-deficiency had a more significant effect (Figure 3-5b). Double knockout B cells also displayed mildly deficient SDF-1-induced migration although more cells migrated as compared to *Rasgrp3*$^{-/-}$ B cells. The results from *Rasgrp3*$^{-/-}$ and double knockout mice suggest that RasGRP3 is not essential for Rap activation and function in B cells.

3.3 RasGRP3 deficiency suppresses *Rasgrp1*$^{-/-}$ autoimmunity

I examined several immunological parameters in RasGRP1- and RasGRP3-deficient mice to determine the impact of defective BCR-Ras signaling on the immune system. In young mice, spleen size was similar in all four genotypes. As reported previously (Layer et al. 2003), *Rasgrp1*$^{-/-}$ mice develop splenomegaly at 2-3 months of age (Figure 3-6a). This effect apparently arises from a defect in T cell development, which by an unknown mechanism leads to autoimmunity (see section 1.6.6). Surprisingly, splenomegaly was never observed in aged (3-6 months old) double mutant mice, nor was it observed in *Rasgrp3*$^{-/-}$ mice. The suppression of this *Rasgrp1*$^{-/-}$ phenotype by the *Rasgrp3* null mutation was also apparent from the numbers of cells recovered per spleen (Figure 3-6b).

To gain a better understanding of this suppression, serum immunoglobulin levels were studied in order to assess B cell function in aged, non-immunized mice. As reported previously (Layer et al. 2003), aged *Rasgrp1*$^{-/-}$ mice exhibited elevated titers of most immunoglobulin isotypes as compared to wildtype mice (Figure 3-7). These antibodies include species that give the strong anti-nuclear staining pattern characteristic of lupus-like autoimmune diseases (Figure 3-8a). In contrast, serum from *Rasgrp3*$^{-/-}$ mice exhibited lower titers of IgG1 and IgG2a, while other isotypes were present at concentrations equivalent to that observed in wildtype mice. Furthermore, anti-nuclear antibodies were not evident in RasGRP3-deficient mice. Double mutant mice also lacked anti-nuclear antibodies, consistent with *Rasgrp3*$^{-/-}$ acting as a *Rasgrp1*$^{-/-}$ suppressor mutation in terms of autoimmunity. Digitally adjusting the brightness of HEp2a immunofluorescence photographs demonstrated that serum from double knockout mice display a diffuse cytoplasmic staining (Figure 3-8b). This staining pattern indicates that the lack of detectable anti-nuclear antibodies in these mice is not simply an effect of decreased antibody levels. Intriguingly, immunoglobulin titers of double mutant mice were higher than titers of *Rasgrp3*$^{-/-}$ mice. When sera from young mice of all four genotypes were tested, no anti-nuclear antibodies were observed (Figure 3-8c). This confirms the late-onset nature of the autoimmunity and suggests that autoimmunity was not present in mice for which BCR signaling experiments were performed.

The suppression of the *Rasgrp1* mutant autoimmunity in the double mutant mice prompted me to perform an experiment to verify the T cell-dependence of the disease. Nude mice (*nu/nu*) lack a thymus and thus are T cell-deficient although they have relatively normal numbers of B cells. In aged *Rasgrp1*$^{-/-}$*; nu/nu* mice, spleens were not enlarged and anti-nuclear antibodies were not evident in their sera (Figure 3-8d and e). Interestingly, splenomegaly was not consistently observed in aged *Rasgrp1*$^{-/-}$ mice on the C57BL/6J genetic background. However, in these mice, as with mice on the mixed 129/J; C57BL/6J genetic background, anti-nuclear antibodies were observed in all aged mice tested.

3.4 B cell development does not require RasGRP1 or RasGRP3

I wished to determine if the lack of BCR-induced Ras activation in double knockout mice causes a defect in B cell development as it has been reported that Ras activation is required for this process (see section 1.7.3). Using surface marker analysis of bone marrow cells, I was unable to discern any profound effect of RasGRP1; RasGRP3 deficiency on the development of B cells from common lymphoid progenitor (CLP) to pro-B, pre-B and mature-B cells (Figure 3-9a and b). The total number of splenic B cells was also normal in these mice (B220^{+} in Figure 3-9e).

I next looked at splenic B cell development. The relative ratios of T1 (immature), T2 (intermediate) and M (mature) B cell developmental stages (Carsetti et al. 2004) in the spleen were not affected by the absence of RasGRP1 and/or RasGRP3 (Figure 3-9c and d), with the variability between genotypes being less than that within a given genotype. One difference I did observe was the increased number of B220$^+$ IgM$^-$ IgD$^-$ B cells in the spleen of *Rasgrp1*$^{-/-}$ mice. There are normal numbers of mature B220$^+$ CD21/35$^+$ IgM$^-$ B cells, so it is likely that the IgM$^-$ IgD$^-$ cells are class-switched mature B cells expressing another immunoglobulin isotype. This likely reflects the initial stages of autoimmunity in these mice, as this population is not increased in double knockout mice. Additionally, because the class-switched B cells are a mature cell population they do not represent a defect in B cell development. Of note, these studies were performed on the mixed 129/J; C57BL/6J background and genetic background effects may make identification of minor B cell development differences difficult due to possible animal-to-animal variability. The majority of the B cells in the spleen are B2 cells (CD5$^-$) while a small proportion are B1 cells (CD5$^+$). *Rasgrp3*$^{-/-}$ mice have wildtype numbers of B1 cells while this population is greatly reduced in both *Rasgrp1*$^{-/-}$ and double knockout mice (Figure 3-9e). This indicates a developmental defect in a small subpopulation of splenic B cells.

Young and aged *Rasgrp1*$^{-/-}$ mice both have an increase in the proportion of CD138$^+$ (syndecan-1) cells reflecting a larger population of antibody secreting plasma cells (Figure 3-10a and b). The likelihood that this population contributes to autoimmunity in the *Rasgrp1*$^{-/-}$ mice is highlighted by the observation that, as with IgM$^-$ IgD$^-$ B cells, *Rasgrp1*$^{-/-}$ mice have at least 3 times more CD138$^+$ cells than do double mutant mice. A complementary result was observed with *Rasgrp1*$^{-/-}$*; nu/nu* mice which have a greatly reduced CD138$^+$ population and lack autoimmunity (Figure 3-10c). However, the presence of increased CD138$^+$ cells alone is not diagnostic of disease as anti-nuclear antibodies are not present in 6 week old *Rasgrp1*$^{-/-}$ mice or aged double knockout mice. Compared to wildtype, these mice have a 6-fold and a 2-fold increase in CD138$^+$ cells respectively (Figures 3-8a-c and 3-10a plus b). The elevated level of CD138$^+$ cells in double knockout mice compared to wildtype of the same age correlates with the increased antibody levels observed in these mice (Figures 3-7 and 3-10b).

As reported previously, young *Rasgrp1*$^{-/-}$ mice have very few splenic T cells, reflecting a failure of positive selection in the thymus (Figure 3-10d). The few T cells that do survive development eventually give rise to a large autoreactive and mostly CD4^{+} T cell population that is implicated in the autoimmune disease (Figure 3-10e)(Layer et al. 2003). *Rasgrp3*$^{-/-}$ mice have normal numbers of CD4^{+} and CD8^{+} splenic T cells, consistent with my observation that T cells do not express this protein (Figure 3-10d and e). Notably, older double mutant mice have a lower proportion of CD4^{+} T cells than autoimmune *Rasgrp1*$^{-/-}$ mice of a similar age, indicating that suppression of the autoimmunity occurs prior to the expansion of this population. The CD4^{+} population in double knockout mice does, however, increase as the mice age although by 6 months it still is far smaller than wildtype. This may indicate that autoimmunity in double knockout mice is due to far later onset than *Rasgrp1*$^{-/-}$ mice rather than being a case of complete suppression. Mice over 6 months of age have not been tested. Judging by the slow increase in CD4^{+} T cells in double knockout mice, it is not clear if these mice would get autoimmunity before they die other causes.

3. 5 RasGRP1 and RasGRP3 deficiencies affect humoral immune responses

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I used the hapten-carrier system to determine the consequence of defective BCR-Ras signaling on antibody responses following immunization. A hapten is a small compound that can be bound by the BCR but does not induce an immune response unless conjugated to another molecule. T-dependent (TD) antigens require T cell help in order to generate an effective response (see section 1.7.5). The immunogen is a hapten conjugated to a protein. T-independent type I (TI-I) responses utilize a polyclonal activator of B cells, such as lipopolysaccharide conjugated to a hapten, while T-independent type II (TI-II) responses use large repetitive hapten-conjugated molecules to crosslink the BCR.

Compared to wildtype, *Rasgrp1*$^{-/-}$ mice were able to generate similar levels of hapten-specific antibodies in the TD protocol (Figure 3-11). Depending on the isotype, *Rasgrp1*$^{-/-}$ mice produced equal or even greater titers in the TI-I and TI-II responses. In particular, hapten-specific IgG1 and IgG2a levels were higher in the *Rasgrp1*$^{-/-}$ mutant under both protocols, possibly reflecting a constitutive T cell influence that is not normally elicited by this injection protocol. That is to say, the *Rasgrp1*$^{-/-}$ T cells may give constitutive B cell help that allows a TI response to resemble a TD response.

Rasgrp3$^{-/-}$ mice were able to generate antibody responses in each immunization protocol although they were not as strong as wildtype, with the production of several isotypes either greatly diminished or absent (Figure 3-11). As expected from my signaling results, the double mutant mice exhibited a more severe defect in antibody production in both TD and TI-I immunizations as compared to either single mutant, although the effect was again isotype-specific. This antibody production deficit was apparent in both IgG1 and IgG2a production during the TD response. However, this IgG1 and IgG2a defect was more severe in the TI-I response with an almost complete lack of hapten-specific antibodies of these isotypes in the serum. The greater defect in the TI-I response suggests that the abnormal RasGRP1-deficient T cells are able to rescue partially the production of these two isotypes. Hapten-specific IgM, IgG2b, and IgG3 levels were relatively normal in mice of all genotypes in both TD and TI-I responses indicating that the immunoglobulin secretion defect of double knockout mice is isotype specific. TI-II immunizations generated much weaker immune responses. *Rasgrp3*$^{-/-}$ mice were deficient in production of all isotypes in the TI-II response while *Rasgrp1*$^{-/-}$ mice produced elevated levels of most isotypes. In this immunization protocol, double knockout mice generate elevated immunoglobulin levels as compared to RasGRP3-deficient mice suggesting an influence of autoimmune T cells.

3.6 RasGRP1 and RasGRP3 are required for BCR-induced proliferation

Cell division is required for immunoglobulin class-switching and B cell differentiation into antibody secreting cells. As such, a lack of proliferation would manifest as a defective humoral immune response (see section 1.7.5). Furthermore, T cells that lack RasGRP1 exhibit reduced proliferative responses (Dower et al. 2000; Priatel et al. 2002; Layer et al. 2003). Accordingly, I examined the roles of RasGRP1 and RasGRP3 in B cell proliferation induced by various stimuli *ex vivo*.

B cells from young mice of all four genotypes were labeled with 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured with or without stimulation for four days. Monitoring mitotic dilution of CFSE fluorescence by flow cytometry allowed for the determination of the number of cell divisions undergone by each cell in addition to providing a measure of both the percentage of cells that divided and the average number of cell divisions for the population as a whole. In all genotypes, treatment with anti-IgM antibodies or IL-4 alone was ineffective at inducing proliferation while in combination they were able to cause significant cell division of wildtype B cells (Figure 3-12). This response was largely intact in RasGRP1 single mutant B cells, dramatically affected by the loss of RasGRP3 and totally absent in the double mutant cells. These defects in proliferation of mutant B cells parallel the results of my biochemical analysis of BCR-Ras-Erk signaling (Figure 3-2a).

Similar results were obtained when anti-CD40 antibody stimulation was used, with roughly equal proliferation occurring in wildtype and *Rasgrp1*$^{-/-}$ B cells, and a significantly decreased *Rasgrp3*$^{-/-}$ B cell response (Figure 3-12). While anti-CD40 treatment induced significant proliferation of double knockout B cells, this response was greatly reduced as compared to wildtype. Interestingly, when anti-IgM treatment was combined with anti-CD40, double knockout B cells displayed a substantially decreased proliferative response as compared to treatment with anti-CD40 alone. This response is in contrast to wildtype and single mutants that displayed enhanced proliferation when these two stimuli were combined. The proliferation deficiency of double knockout B cells was minimal when anti-CD40 and IL-4 treatment was used, providing sufficiently high concentration of IL-4 was present. Lipopolysaccharide (LPS) was also capable of inducing a similar degree of proliferation of B cells from mice of all genotypes. Thus, RasGRP1 and RasGRP3 are not absolutely required for cell division, rather they are required for cell division induced by certain mitogens only. Interestingly, anti-IgM has an inhibitory effect on LPS-mediated and anti-CD40 plus IL-4-mediated proliferation in all genotypes, but did not completely abrogate the proliferative effect of these stimuli in double knockout B cells, indicating that with sufficient co-stimulation double knockout B cells can still proliferate in the presence of antigen receptor stimulation.

The CFSE-labeling experiments reveal the net effect of proliferation over the course of the experiment. To verify these findings in another system, B cells were incubated for one hour with bromodeoxyuridine (BrdU) to label the fraction of cells actively engaged in DNA replication at the end of the culturing period, followed by analysis of BrdU incorporation by flow cytometry. In this protocol, cells that are actively replicating their DNA (S phase) during the BrdU incubation period can be detected by their increased BrdU staining and by their intermediate 7-AAD labeling (Figure 3-13a). As with the CFSE experiment, single mutant B cells had diminished proliferative responses to anti-IgM plus IL-4 treatment, while double mutant B cells were virtually inactive in this assay (Figure 3-13a and b). While the general pattern of results obtained from the two different assays was the same, one difference was observed. The BrdU method allowed us to detect proliferation induced by anti-IgM alone in wildtype B cells. While *Rasgrp1*$^{-/-}$ B cells responded normally to this treatment there was a complete lack of a proliferative response in *Rasgrp3*$^{-/-}$ and doubly deficient B cells. Overall, the data fit the idea that RasGRP3 plays a more important role than RasGRP1 in BCR-induced proliferation, with substantial redundancy evident in most circumstances.

In order to determine if double knockout B cells are capable of activation and differentiation, I further examined *ex vivo* stimulated B cells by flow cytometry. Upon stimulation with anti-CD40 plus IL-4 for four days, CFSE-labeled B cells from mice of all four genotypes were capable of expressing the activation marker CD69, although undivided double knockout B cells failed to do so (Figure 3-14a). This indicates that proliferation and CD69 upregulation are linked. The extensive proliferation from anti-CD40 plus IL-4 treatment allowed for the analysis of B cell class-switching from IgM to IgG1 and differentiation to CD138^{+} plasma cells. Double knockout B cells were capable of undergoing both of these processes although, once again, there was a reduction in these populations that correlated with decreased proliferation (Figure 3-14b and c). Thus, RasGRP1 and RasGRP3 are required for activation and differentiation of B cells, in as much as they are required for proliferation.

While stimulating mutant B cells, I noticed an effect of genotype on B cell survival. Upon treatment with anti-IgM, *Rasgrp3*$^{-/-}$ and double knockout B cells displayed a greatly reduced survival as compared to wildtype as determined by propidium iodide exclusion (Figure 3-15). This viability defect was not as evident when cells were untreated or when IL-4 stimulation was used. Interestingly, *Rasgrp1*$^{-/-}$ B cells displayed reduced survival that was independent of stimulation. Given the decreased viability of B cells from mutant mice, it is important to note that dead cells are gated out of the analysis in the proliferation assays. Nonetheless, the proliferation defects observed could be a result of the death of cells that attempt to divide, while non-dividing cells might not be as susceptible. The decreased viability of *Rasgrp1*$^{-/-}$ B cells does not appear to affect, or be affected by, the proliferative response of these cells.

3.7 Double knockout B cells are less responsive to T cell-mediated stimulation

Loss of RasGRP1 expression causes a T cell development defect that leads to late-onset autoimmunity in a manner that requires RasGRP3 expression, presumably in B cells. To uncover the underlying mechanisms of this autoimmunity and the suppression in double knockout mice, I used co-cultivation assays to study T cell-B cell interactions. *Rasgrp1*$^{+/+}$ and *Rasgrp1*$^{-/-}$ CD4^{+} T cells were isolated, as were B cells of all four genotypes. These cells were CFSE-labeled and then co-cultured in various combinations along with TCR (anti-CD3ε) and BCR (anti-IgM) stimulatory antibodies.

In this assay, substantial proliferation of anti-IgM treated wildtype B cells was observed after co-cultivation with anti-CD3ε stimulated *Rasgrp1*$^{+/+}$ CD4^{+} T cells (Figure 3-16a). Loss of RasGRP1 expression in B cells had no effect on proliferative responses, while *Rasgrp3*$^{-/-}$ B cells underwent fewer cell divisions on average. The proliferation defect was most severe in double knockout B cells with the majority of the cells not dividing. In parallel cultures, *Rasgrp1*$^{-/-}$ CD4^{+} T cells elicited a greater proliferative response from B cells of all genotypes although a similar pattern of RasGRP-dependence on cell division was observed. Most notably, *Rasgrp1*$^{-/-}$ CD4^{+} T cells were capable of inducing a substantial double knockout B cell proliferative response. I also confirmed that *Rasgrp1*$^{-/-}$ CD4^{+} T cells have a decreased proliferative response upon TCR-stimulation as compared to wildtype cells (Figure 3-16b).

RasGRP1-deficient T cells have been shown to secrete elevated levels of IL-4 upon anti-CD3ε stimulation as compared to wildtype (Layer et al. 2003). Therefore, I wished to determine if this cytokine is responsible for the increased B cell proliferation induced by *Rasgrp1*$^{-/-}$ T cells in the co-culture experiments. Cell-free supernatants from cultures of *Rasgrp1*$^{+/+}$ or *Rasgrp1*$^{-/-}$ anti-CD3ε treated CD4^{+} T cell were used to stimulate B cells (Figure 3-17). In response to treatment with *Rasgrp1*$^{+/+}$ supernatants, wildtype B cells showed the greatest proliferation response followed by *Rasgrp1*$^{-/-}$ and then *Rasgrp3*$^{-/-}$ B cells, while double knockout B cells showed no response. Furthermore, media conditioned by *Rasgrp1*$^{-/-}$ T cells induced greater B cell proliferative responses. When an IL-4 neutralizing antibody was included with the conditioned medium, proliferation of B cells was reduced. Notably, this neutralizing antibody abolished the enhanced proliferative capabilities of the supernatants from *Rasgrp1*$^{-/-}$ T cells. These results suggest that *Rasgrp1*$^{-/-}$ CD4^{+} T cells utilize IL-4 to cause enhanced B cell proliferation and that this proliferation is largely RasGRP-dependent.

3.8 RasGRP1 and RasGRP3 are required for BCR induced cyclin D2 expression

D-type cyclins bind and activate the cyclin-dependent kinases cdk4 and cdk6, which in turn phosphorylate and inactive the cell cycle regulator pRB and related proteins. This allows for cell cycle progression and cell division. There are three D-type cyclins of which cyclin D2 and cyclin D3 are expressed in B cells (Chiles 2004). Resting B cells can be stimulated to express cyclin D2 by a combination of anti-IgM and IL-4 (Tanguay et al. 1996; Solvason et al. 2000). Cyclin D2 upregulation by these stimuli has been found to be dependent on PKC and Erk activation in addition to other components downstream of BCR signaling, and blocking this upregulation by the use of inhibitors or mice deficient in these proteins results in defective B cell proliferation (Piatelli et al.

2002; Chiles 2004). Failure of double knockout B cells to proliferate upon anti-IgM plus IL-4 treatment might be a result of defective cyclin D2 upregulation.

Wildtype splenic B cells stimulated with IL-4 displayed transient and relatively weak cyclin D2 upregulation (Figure 3-18a) which correlated with a failure to proliferate (Figures 3-12, and 3-13). A similar result was observed with RasGRP1 and RasGRP3 single and double knockout B cells although in the absence of RasGRP3 there was a somewhat decreased induction. Upon stimulation with anti-IgM there was a late but more significant cyclin D2 upregulation in wildtype B cells (Figure 3-18b) which correlated with a small amount of proliferation (Figure 3-13). The response of *Rasgrp1*$^{-/-}$ B cells was the same as wildtype, however *Rasgrp3*$^{-/-}$ and double knockout B cells failed to induce cyclin D2 upregulation upon anti-IgM treatment (Figure 3-18b). These results again correlated with the proliferation induced by anti-IgM (Figure 3-13). When anti-IgM and IL-4 treatments were combined, cyclin D2 induction was rapid and sustained in wildtype, *Rasgrp1*$^{-/-}$ and *Rasgrp3*$^{-/-}$ B cells (Figure 3-18c). Double knockout B cells failed to induce cyclin D2 under this treatment. Yet again, the cyclin D2 expression results correlated with proliferation results (Figures 3-12 and 3-13).

One way that IL-4 induces gene expression is by causing the phosphorylation and thus activation of STAT6 (see section 1.7.4). Since there is defective synergy between IL-4 and anti-IgM at the level of cyclin D2 upregulation, and STAT6-deficient B cells have defective proliferation (Shimoda et al. 1996; Takeda et al. 1996), I wished to test the status of the IL-4-STAT6 pathway in double knockout B cells to insure that deficiencies in this pathway are not responsible for the defective proliferation observed in double knockout mice. IL-4 treatment resulted in normal phosphorylation of STAT6 in mice of all four genotypes (Figure 3-18d), suggesting that IL-4 signaling is not defective in these mice.

3.9 Summary

Here I showed that RasGRP1 and RasGRP3 are co-expressed in primary murine B cells. I found that RasGRP1 and RasGRP3 play partially redundant roles in Ras activation upon BCR-stimulation and that RasGRP3 is solely required for maintaining a basal level of Ras activation in untreated B cells. RasGRP1 and RasGRP3 are the sole DAG-responsive Ras activators in B cells as, even in an extended time course experiment, PMA was unable to induce Ras-GTP accumulation in double knockout B cells. While PMA induces the downregulation of RasGRP1, this is not the case for RasGRP3. Lack of Ras activation following BCR-ligation in double knockout B cells was a specific result of decreased Ras GEF activity, as B cell surface marker analysis and global tyrosine phosphorylation upon anti-IgM treatment were both normal.

PMA-induced Rap activation in B cells was unaffected by the absence of both RasGRP1 and RasGRP3. SDF-1-induced migration, which is Rap-dependent, was mildly inhibited in double knockout B cells.

While *Rasgrp1*$^{-/-}$ mice develop late-onset autoimmunity characterized by splenomegaly and anti-nuclear antibodies, *Rasgrp1; Rasgrp3* double knockout mice do not. As compared to aged *Rasgrp1*$^{-/-}$ mice, aged double knockout mice exhibited reduced spleen size, immunoglobulin levels, plasma B cells and CD4^{+} T cells in addition to a complete absence of anti-nuclear antibodies. The production of anti-nuclear antibodies in *Rasgrp1*$^{-/-}$ mice was dependent on the presence of T cells.

Deficiency of BCR-ligation induced Ras activation in double knockout mice did not have a significant effect on B cell development in the bone marrow or spleen. In addition, double knockout mice had only mildly defective humoral immune responses, with deficiencies in hapten-specific antibodies observed in only a subset of immunoglobulin isotypes.

RasGRP1 and RasGRP3 are required for proliferation mediated by BCR-stimulation and co-mitogens. Proliferation mediated by non-BCR-stimulation occurs with varying degrees in the absence of RasGRP1 and RasGRP3 expression and certain stimuli, such as LPS, can still induce a normal proliferative response. Treatment of double knockout B cells with anti-CD40 plus IL-4 resulted in only mildly deficient proliferation, activation, class-switching and differentiation indicating that these processes are still functional in these cells. Double knockout B cells also displayed reduced survival compared to wildtype B cells upon anti-IgM treatment although there was a much less significant difference when IL-4 treatment was used. In addition, *Rasgrp1*$^{-/-}$ B cells had decreased survival that was independent of the stimulus used.

Rasgrp1$^{-/-}$ CD4^{+} T cells are capable of inducing greater B cell proliferation as compared to wildtype CD4^{+} T cells. Double knockout B cells are far less responsive to T cell-mediated stimulation, including that promoted by *Rasgrp1*$^{-/-}$ CD4^{+} T cells. Using blocking antibodies, I determined that the enhanced B cell proliferation induced by *Rasgrp1*$^{-/-}$ T cells was mediated, at least in part, by IL-4. While IL-4 signaling to STAT-6 was normal in B cells of all four genotypes, IL-4 failed to synergize with anti-IgM to induce cyclin D2 upregulation in double knockout mice. Thus, the failure of cyclin D2 induction is likely the cause of the RasGRP1; RasGRP3 double deficient B cell proliferative defect.

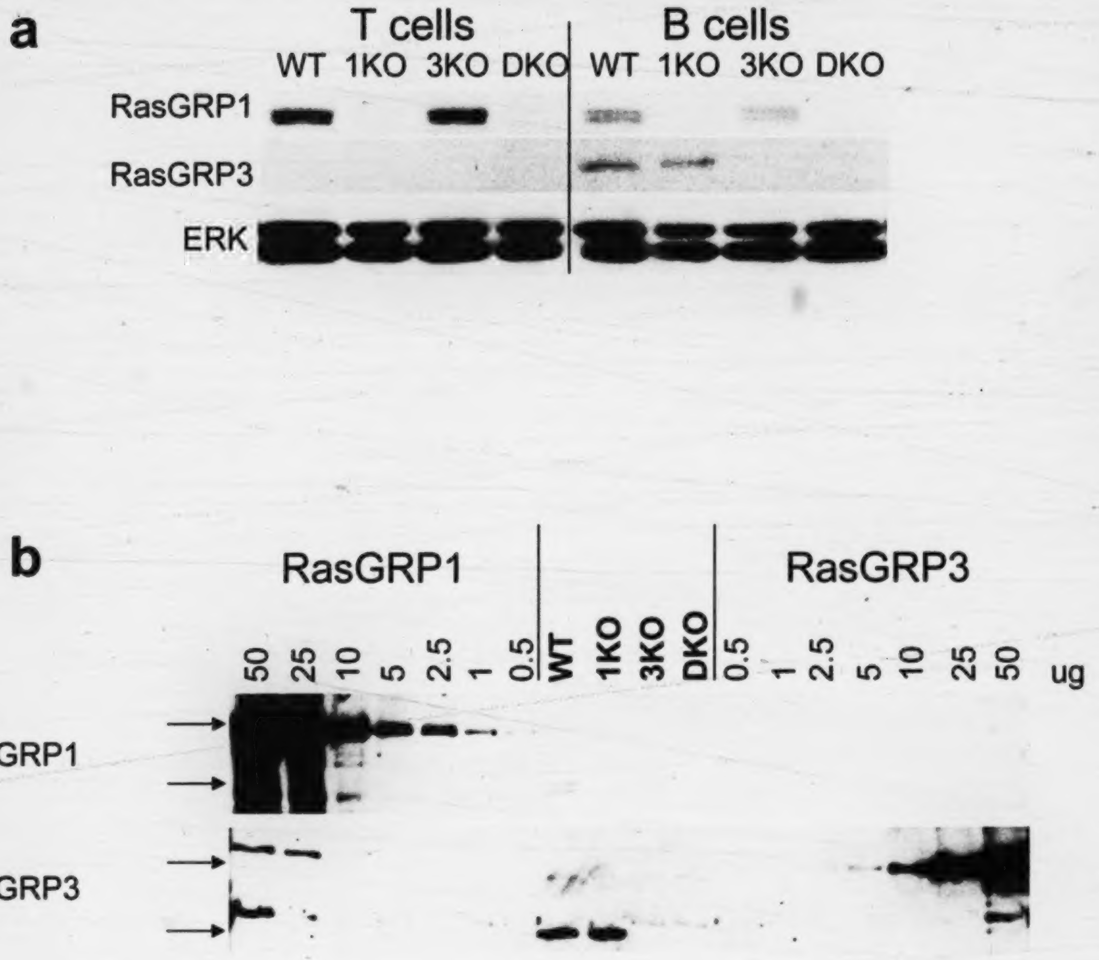
a
T cells
B cells
WT
1KO
3KO
DKO
WT
1KO
3KO
DKO
RasGRP1
RasGRP3
ERK
b
RasGRP1
RasGRP3
50
25
10
5
2.5
1
0.5
WT
1KO
3KO
DKO
0.5
1
2.5
5
10
25
50
ug
RasGRP1
RasGRP3

Figure 3-1. RasGRP1 and RasGRP3 are expressed in B cells.
(a) Splenic T and B cells were prepared from mice of the indicated genotypes and total cell lysates were subjected to immuno-blotting with anti-RasGRP1, anti-RasGRP3 and anti-Erk (loading control) antibodies. Genotypes are: wild-type (WT), $Rasgrp1^{-/-}$ (1KO), $Rasgrp3^{-/-}$ (3KO), and $Rasgrp1^{-/-}$; *Rasgrp3-/-* double mutant (DKO). **(b)** Splenic B cells (3 x 10^5) of the indicated genotypes were lysed and run on SDS-PAGE along with known quantities (values in ug) of recombinant MBP-tagged RasGRP1 and RasGRP3 (provided by Yong Zheng). Immuno-blotting was then performed with anti-RasGRP1 followed by anti-RasGRP3. Arrows indicate the recombinant (upper) and endogenous (lower) proteins of interest.

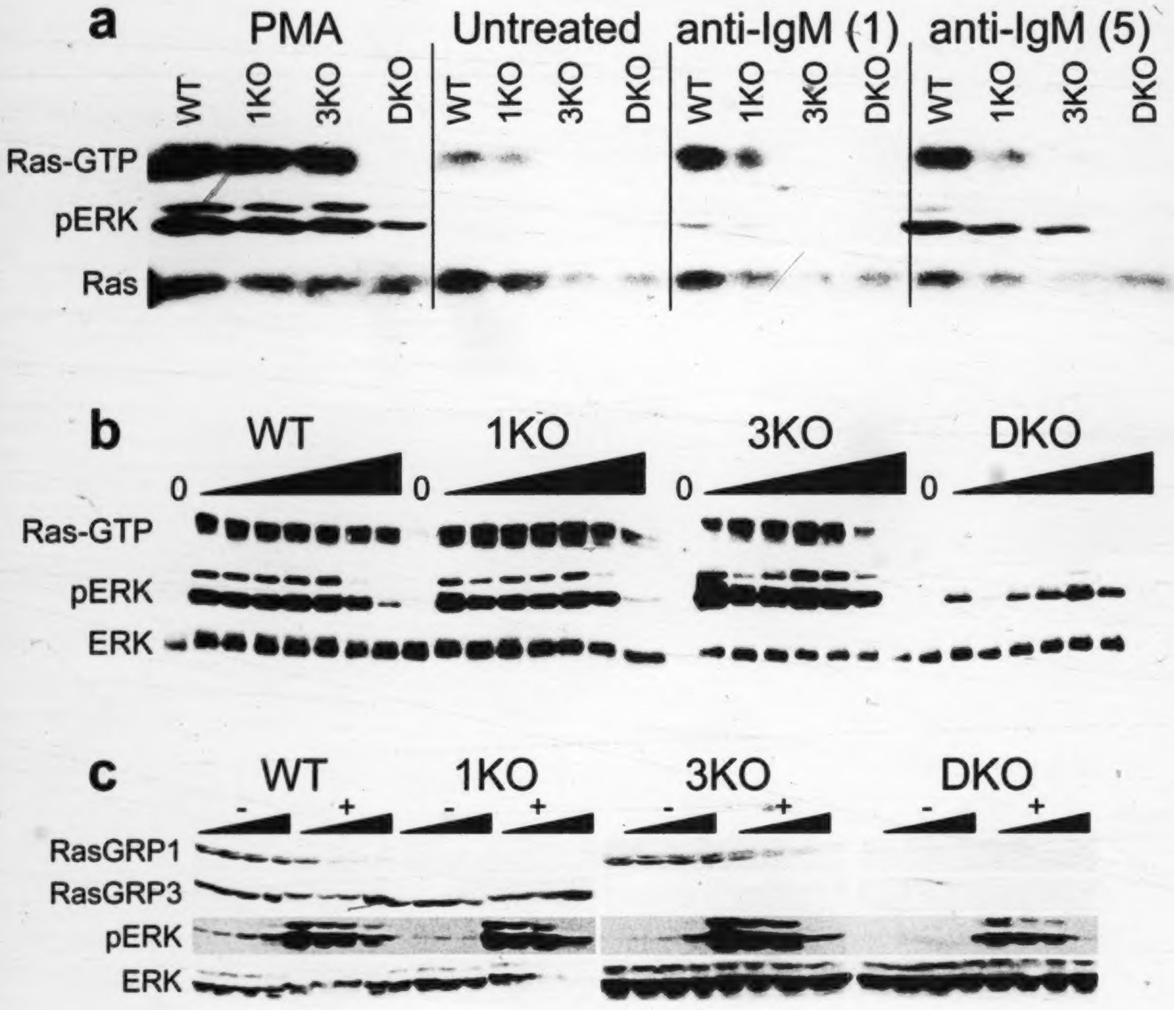
a
PMA
Untreated
anti-IgM (1)
anti-IgM (5)
WT
1KO
3KO
DKO
Ras-GTP
pERK
Ras
b
WT
1KO
3KO
DKO
0
Ras-GTP
pERK
ERK
c
WT
1KO
3KO
DKO
-
+
RasGRP1
RasGRP3
pERK
ERK

Figure 3-2. RasGRP1 and RasGRP3 activate Ras upon BCR-ligation.
(a) Splenic B cells isolated from 6 week old mice of indicated genotypes were left untreated, treated with PMA for 10 minutes or with anti-IgM for 1 or 5 minutes, as indicated. Cell lysates were assayed for Ras-GTP, phospho-ERK, and total Ras. Results are representative of three experiments. **(b)** Red blood cell depleted splenocytes from mice of the indicated genotypes were treated with LPS to enrich B cells. Three days later equal numbers of cells were treated with PMA for 0, 1/12, 1/6, 1/4, 1/2, 1, 4, or 24 hours. Cell lysates were assayed for Ras-GTP, phospho-ERK, and total Erk. **(c)** B cells prepared as in (b) were left untreated (-) or treated with PMA (+) for 1/6, 1, 3, or 22 hours. Cell lysates were assayed for RasGRP1, RasGRP3, phospho-ERK, and total Erk. Genotypes are indicated as in Fig. 3-1.

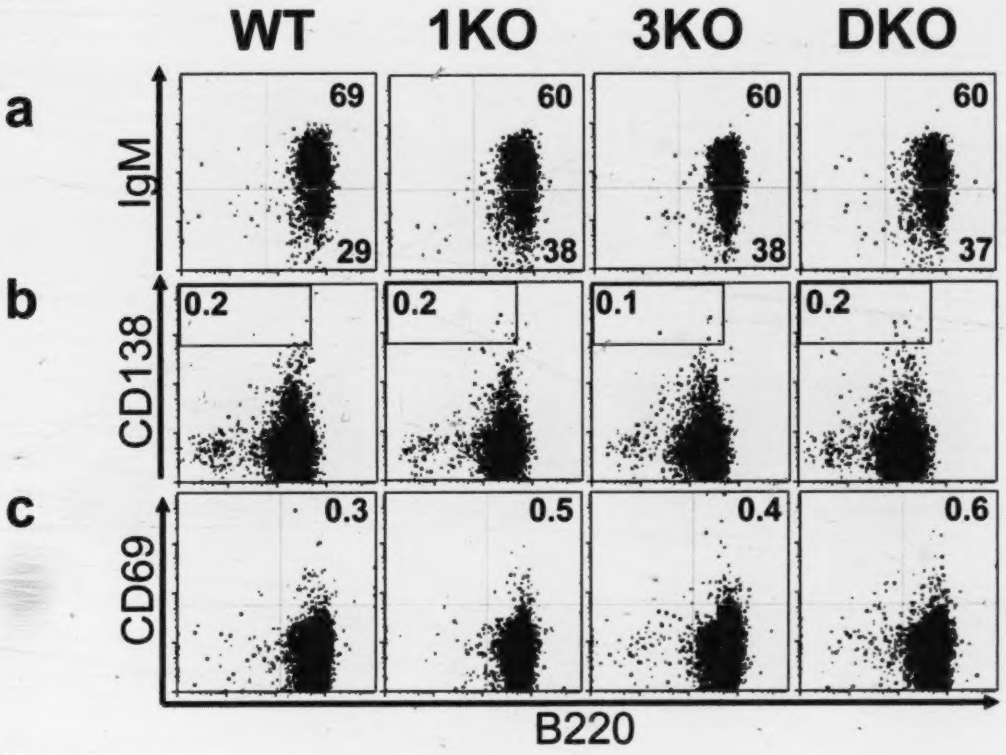

Figure 3-3. Splenic B cells isolated from mice of all four genotypes are similar as determined by flow cytometry.
(a-c) Splenic B cells from 6 week old mice of indicated genotypes were stained with antibodies as indicated. Numbers indicate the percentage of the total cell population in particular region. Genotypes are indicated as in Fig. 3-1.

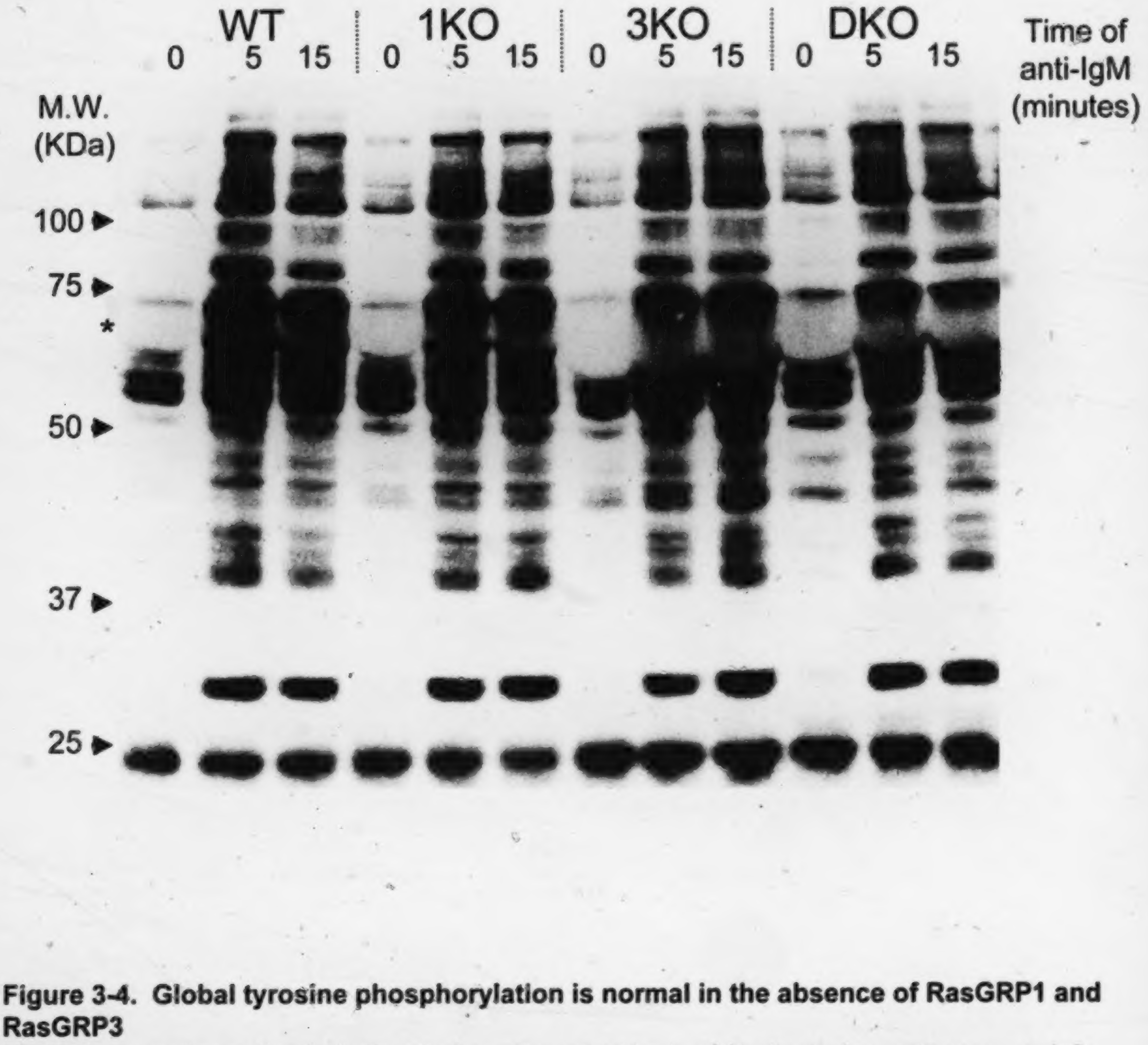
WT
1KO
3KO
DKO
0
5
15
0
5
15
0
5
15
0
5
15
Time of anti-IgM (minutes)
M.W. (KDa)
100
75
*
50
37
25

Figure 3-4. Global tyrosine phosphorylation is normal in the absence of RasGRP1 and RasGRP3
Equivalent numbers of splenic B cells from 6 week old mice of indicated genotypes were left untreated or treated with anti-IgM, as indicated. Cell lysates were probed for phosphotyrosine. Molecular weight markers are shown on left. The asterisk (*) indicates a zone of distortion in the gel that arose from bovine serum albumin contained in the B cell purification buffer. Genotypes are indicated as in Figure 3-1

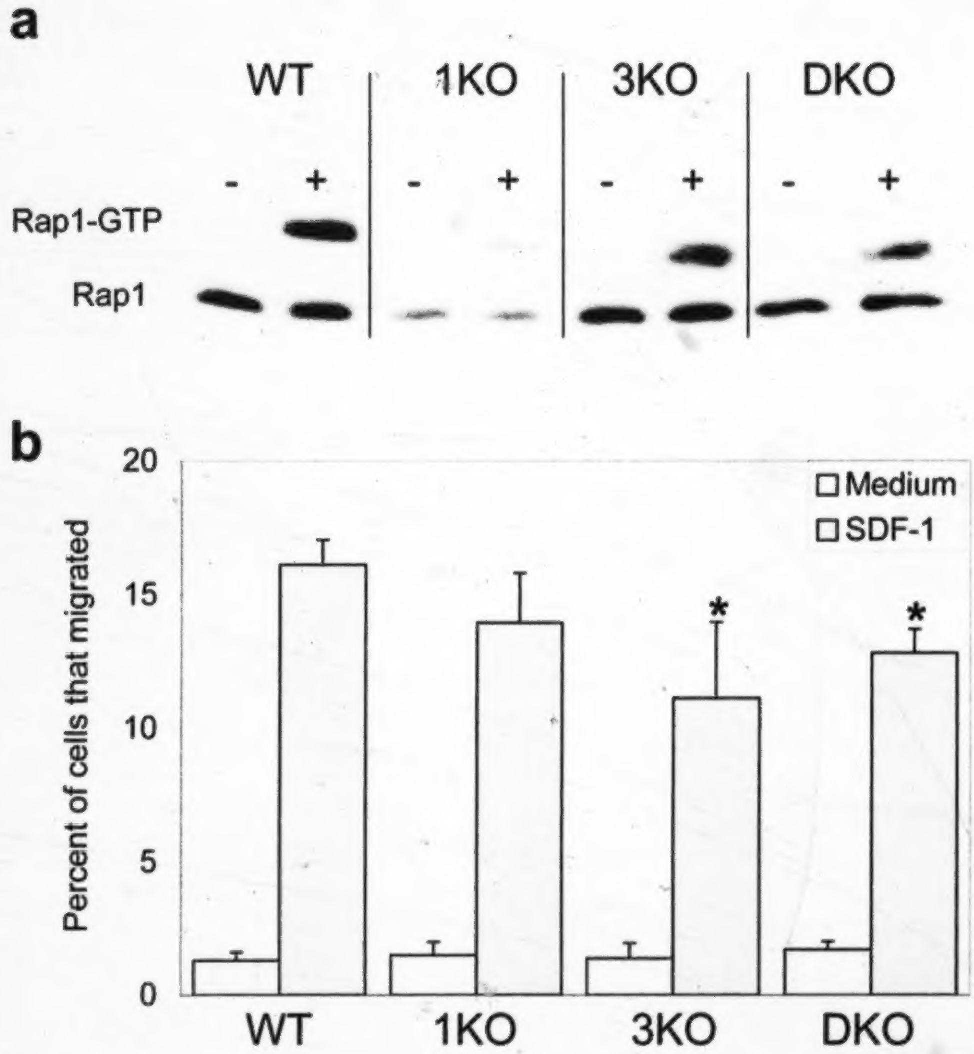

Figure 3-5. Rap activation and function in the absence of RasGRP1 and RasGRP3. **(a)** Red blood cell depleted splenocytes from mice of the indicated genotypes were treated with LPS to enrich B cells. Three days later equal numbers of cells were left untreated (-) or treated with PMA for 10 minutes (+). Cell lysates were assayed for Rap-GTP and total Rap. **(b)** SDF-1-induced migration of isolated splenic B cells was assessed using transwell assays. Indicated is the average percentage of cells that migrated from the upper chamber to the lower chamber in 3 hours at 37^{0}C. The lower chamber contained either medium alone (white bars) or medium supplemented with SDF-1 (grey bars). Error bars are the standard error of the mean from three independent experiments. Genotypes are indicated as in Figure 3-1. Asterisks indicate values that were determined to be statistically different from wildtype using the student t-test ($p<0.05$).

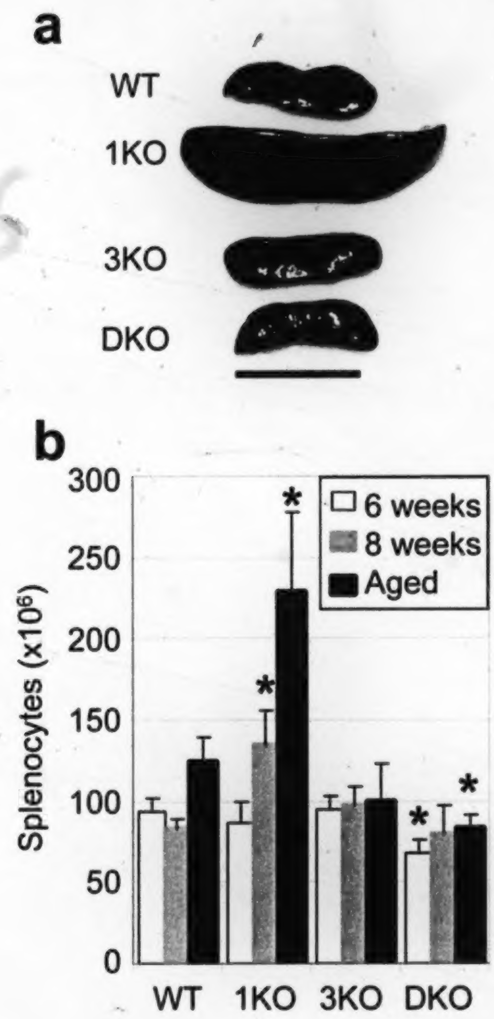

Figure 3-6. Loss of RasGRP3 expression results in suppression of the splenomegaly associated with *Rasgrp1*$^{-/-}$.
(a) Spleens from 4-month-old mice are compared. Bar indicates 1 cm. **(b)** Single cell suspensions were prepared from the spleens of 6 week old, 8 week old and 3-6 months-old ("aged") mice and cells were counted. Genotypes are indicated as in Figure 3-1. Asterisks indicate values that were determined to be statistically different from wildtype using the student t-test ($p<0.05$).

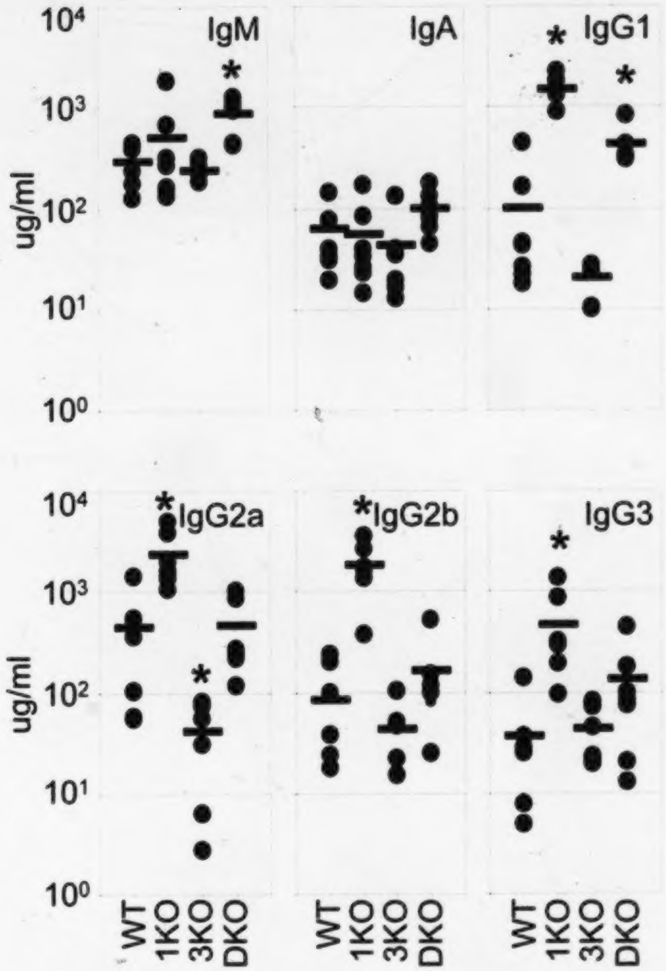

Figure 3-7. RasGRP3-deficiency results in isotype-selective hypogammaglobulinemia and partially suppresses hypergammaglobulinemia associated with *Rasgrp1*$^{-/-}$. Sera were collected from aged mice and antibody isotype concentrations were determined. Each circle represents an individual mouse and a bar represents the mean. Genotypes are indicated as in Figure 3-1. Asterisks indicate values that were determined to be statistically different from wildtype using the student t-test ($p<0.05$).

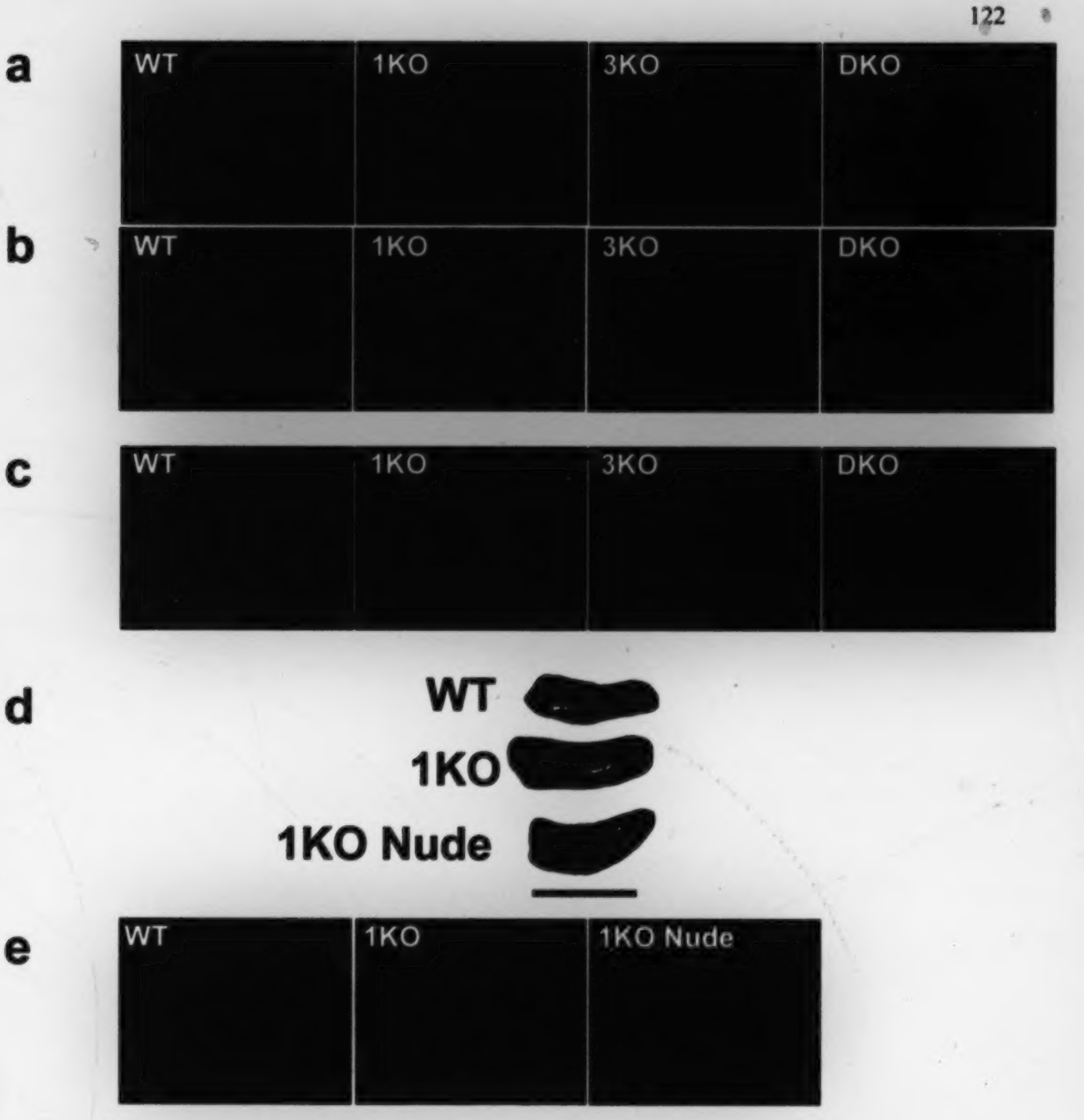
a
WT
1KO
3KO
DKO
b
WT
1KO
3KO
DKO
c
WT
1KO
3KO
DKO
d
WT
1KO
1KO Nude
e
WT
1KO
1KO Nude

Figure 3-8. RasGRP3 expression and T cells are required for production of anti-nuclear antibodies associated with *Rasgrp1*$^{-/-}$ autoimmunity.
(a-c) Sera from aged (a and b) and young (c) mice of the indicated genotypes were tested for anti-nuclear antibodies using HEp-2 cells. Data are representative of five experiments. Photographs in (b) are the same as in (a) but with digitally elevated brightness and contrast levels. **(d)** Spleens from aged mice of the indicated genotypes. Bar indicates 1 cm. **(e)** Sera collected from aged mice of the indicated genotype were tested for anti-nuclear antibodies. Genotypes are indicated as in Figure 3-1 with the exception of *Rasgrp1*$^{-/-}$*; nu/nu* mice (1KO Nude).

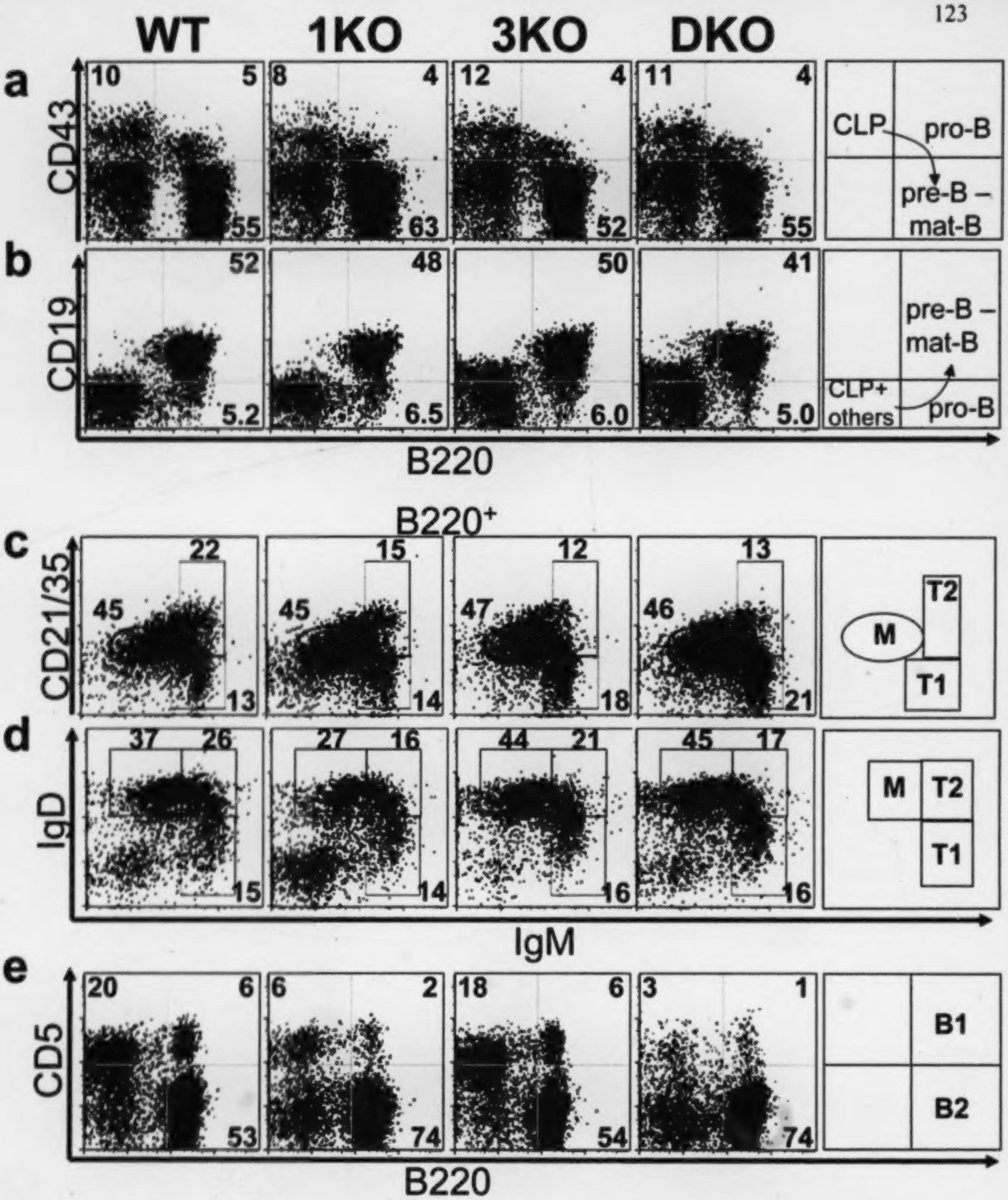

Figure 3-9. B cell development unaffected by the absence of RasGRP1 and/or RasGRP3.

Cells from the bone marrow **(a and b)** or spleen **(c-e)** were collected from 6 week old mice and were stained with antibodies as indicated. Populations are identified according to the standard B cell classification scheme (see text). **(c and d)** The results were obtained by gating B220$^+$ cells. Numbers indicate the percentage of the total cell population in particular region. Genotypes are indicated as in Fig. 3-1.

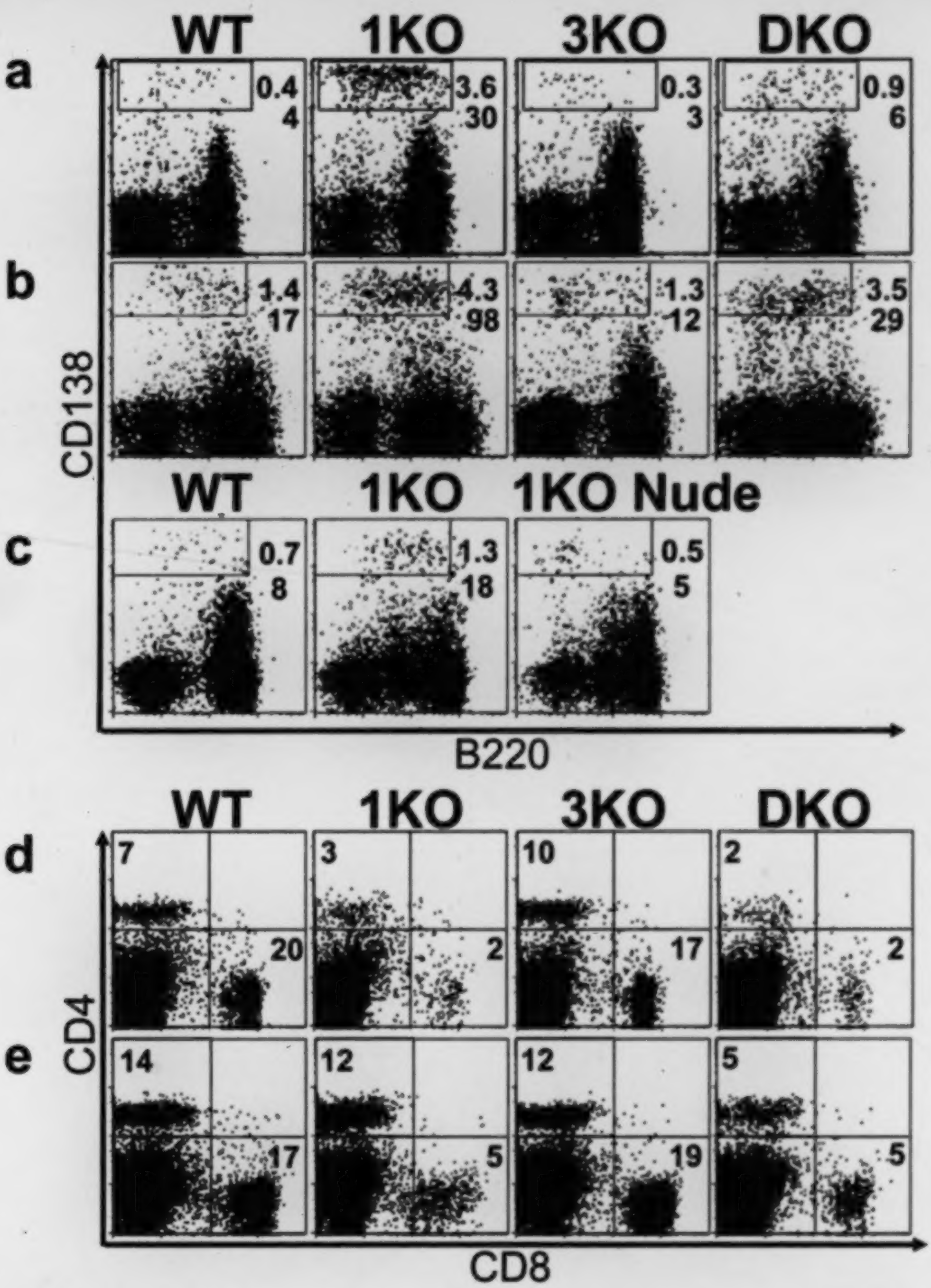

Figure 3-10. RasGRP3 expression is required for the expansion of autoimmune populations.
Total splenocytes from 6 week old (**a and d**) or 4 month old (**b, c, and e**) mice were stained with antibodies as indicated and analyzed by flow cytometry. The results of gating total live splenocytes are plotted. Numbers indicate the total number of cells in particular region (x10^5, bottom number for a-c) or the percentage of the total cell population in particular region. Genotypes are indicated as in Figure 3-1 with the exception of *Rasgrp1*$^{-/-}$; *nu/nu* mice (1KO Nude).

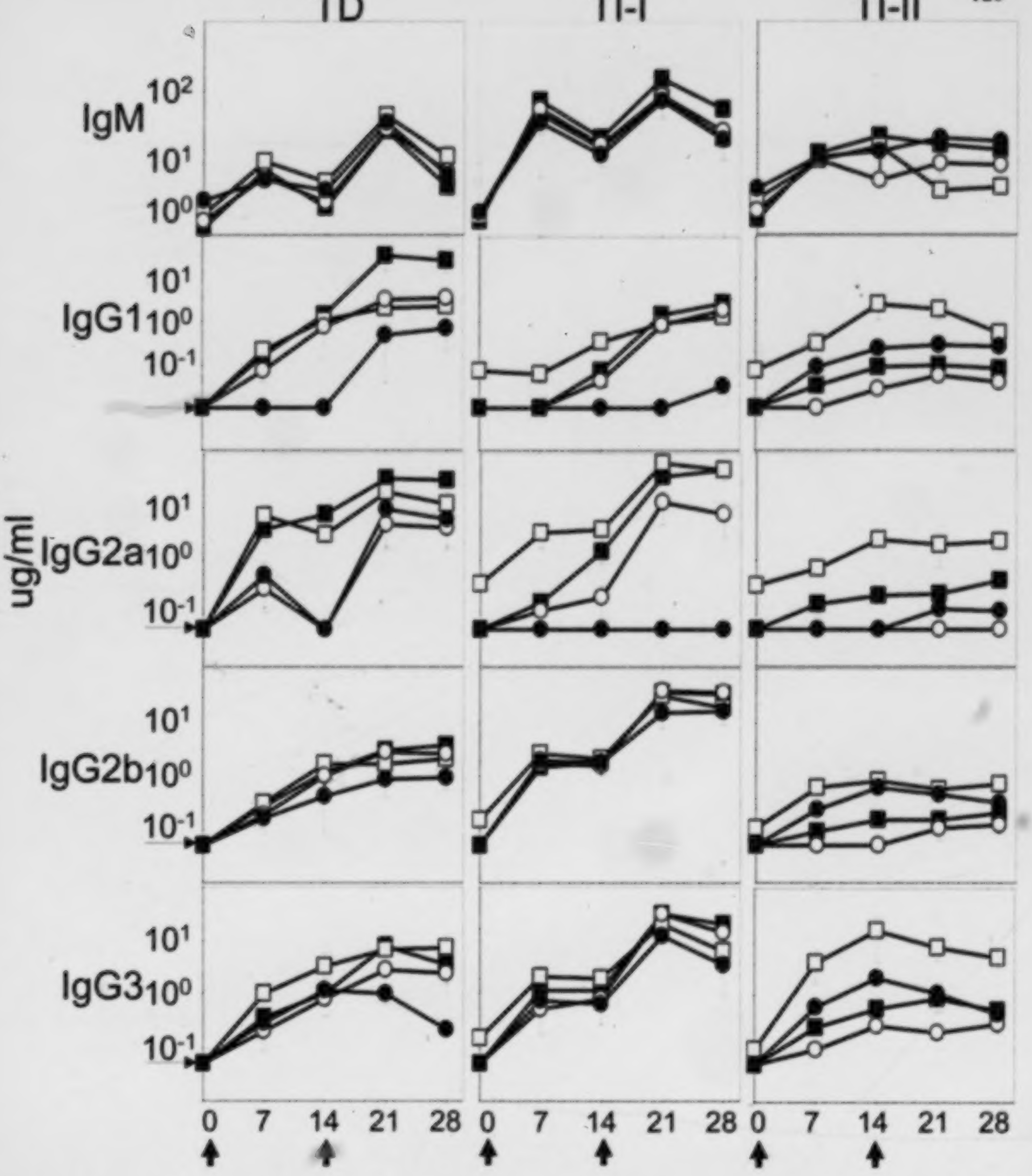

Figure 3-11. Impaired humoral immune responses in *Rasgrp1*; *Rasgrp3* double mutant mice.

Six week old mice of the indicated genotype were immunized with either TNP-KLH (T-dependent, TD), TNP-LPS (T-independent type I, TI-I) or DNP-Ficoll (T-independent type II, TI-II) on day 0 and day 14 (indicated with solid arrows). Serum samples were collected on days indicated and tested for hapten specific antibodies of the given isotypes. Values are mean of 3-6 mice with error bars representing the S.E.M. Dashed arrow represents value below level of detection. Results shown for WT (■), 1KO (□), 3KO (○), and DKO (●) mice. Genotypes are indicated as in Figure 3-1.

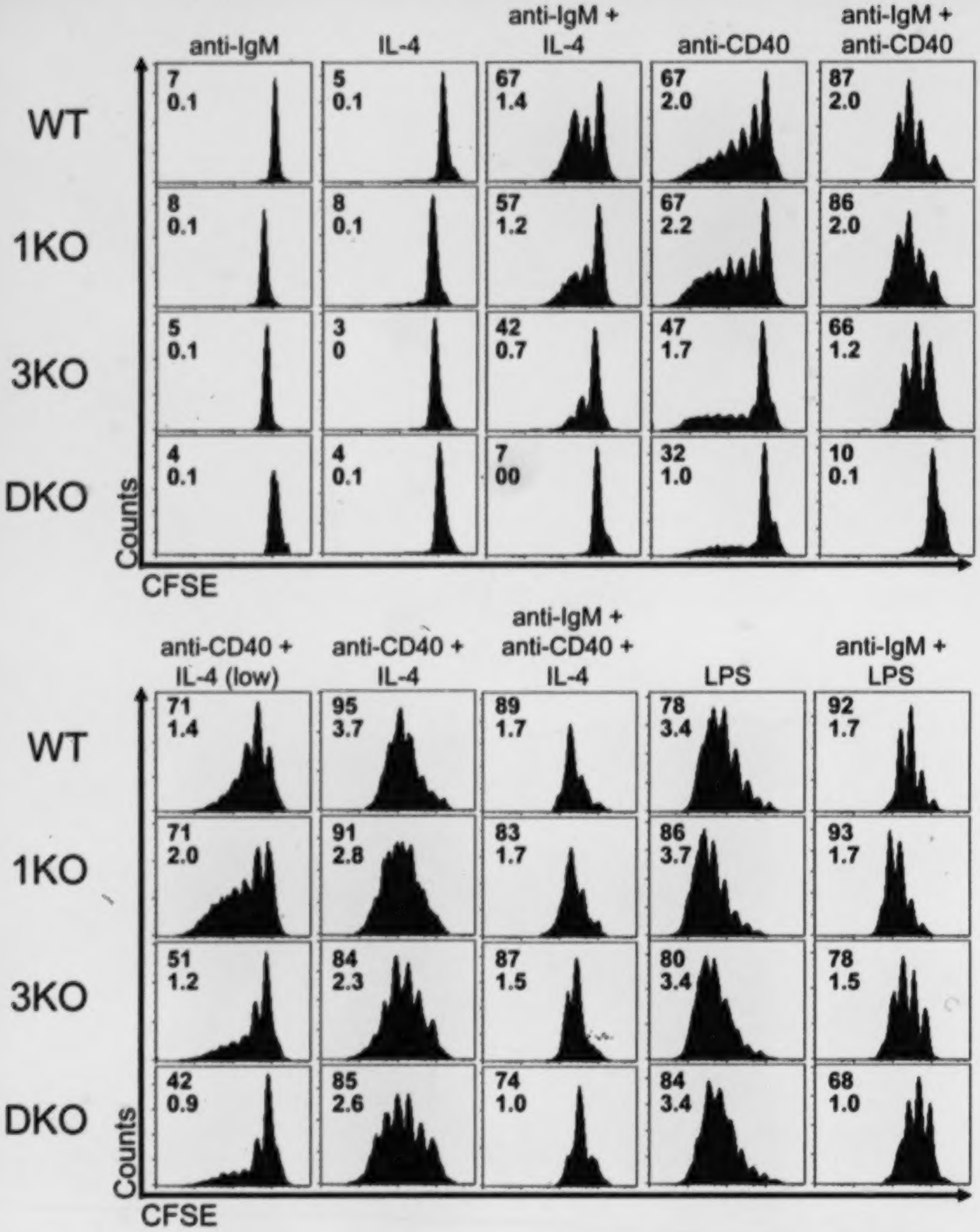

Figure 3-12. Impaired proliferation in the absence of RasGRP1 and RasGRP3 expression.

Splenic B cells were labeled with CFSE and then treated for four days as indicated. Cells were then analyzed by flow cytometry. The percent of cells that divided at least once (upper) and average number of cell divisions (lower) are indicated for each sample. IL-4 was used at 10 ng/ml unless otherwise noted (low, 0.1 ng/ml). Genotypes are indicated as in Figure 3-1.

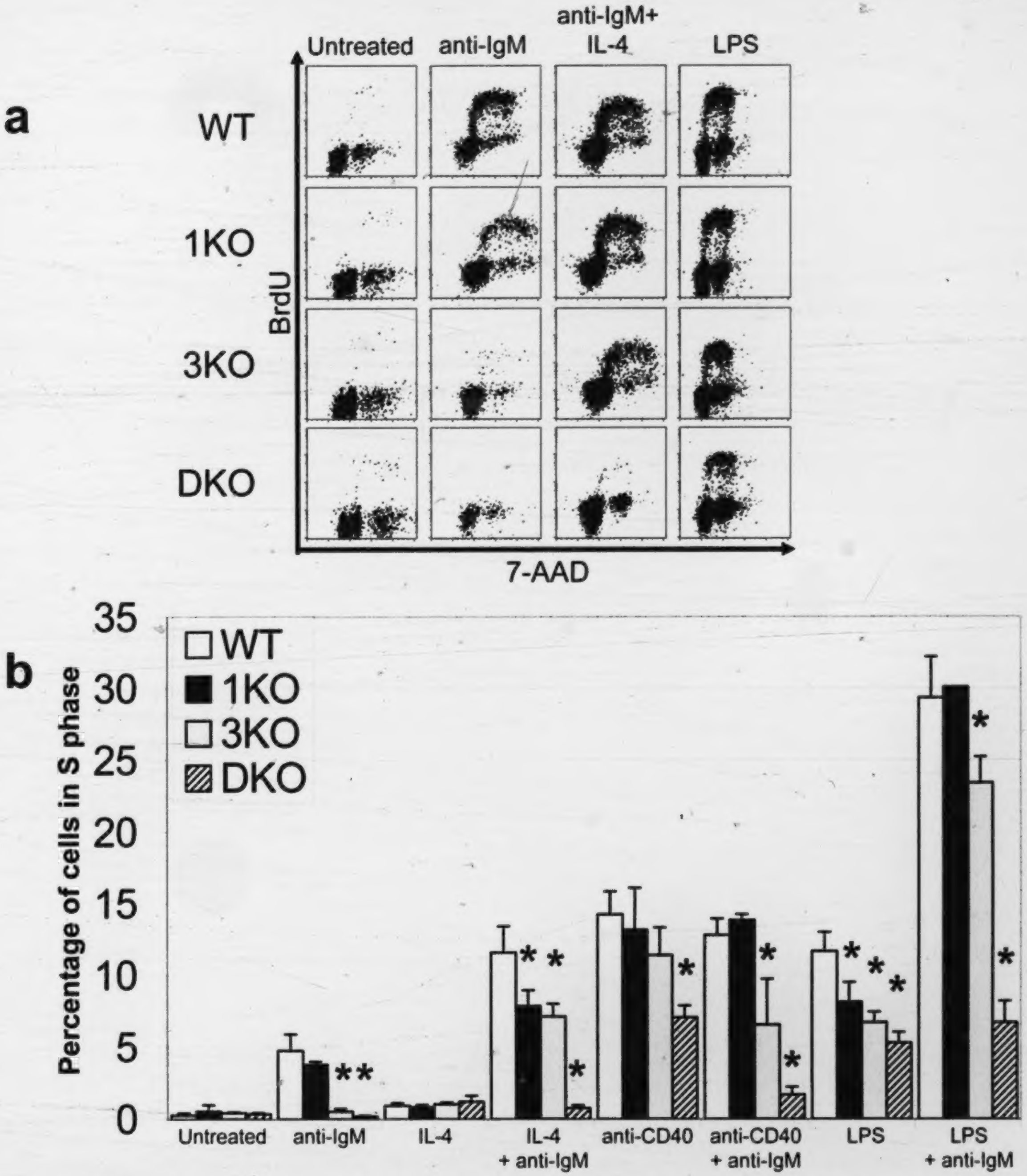

Figure 3-13. Fewer S phase cells upon stimulation in the absence of RasGRP1 and RasGRP3 expression.
Splenic B cells were treated for three days as indicated, followed by BrdU incorporation, antibody staining and flow cytometry analysis. **(a)** Representative data. **(b)** The percentages of B cells that were in S phase after various treatments were determined by flow cytometry. Error bars represent the S.E.M. Genotypes are indicated as in Figure 3-1. Asterisks indicate values that were determined to be statistically different from wildtype using the student t-test ($p<0.05$).

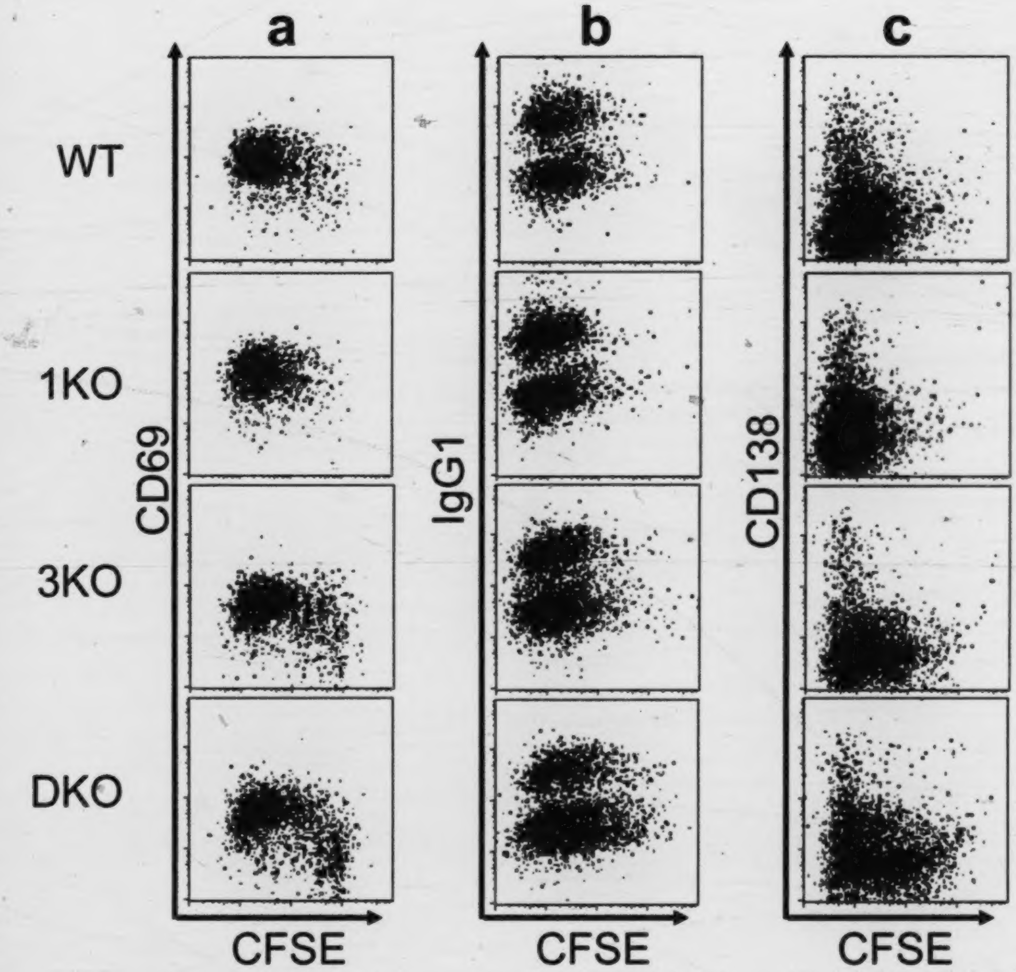

Figure 3-14. B cells deficient in RasGRP1 and RasGRP3 expression are capable of activation, class-switching and differentiation into plasma cells.
Splenic B cells were labeled with CFSE and then treated with anti-CD40 and IL-4 (10 ng/ml) for four **(a)** or five **(b, c)** days as indicated. Cells were then either fixed and permeabilized (b) or directly labeled with fluorescent antibodies and analyzed by flow cytometry. Genotypes are indicated as in Figure 3-1.

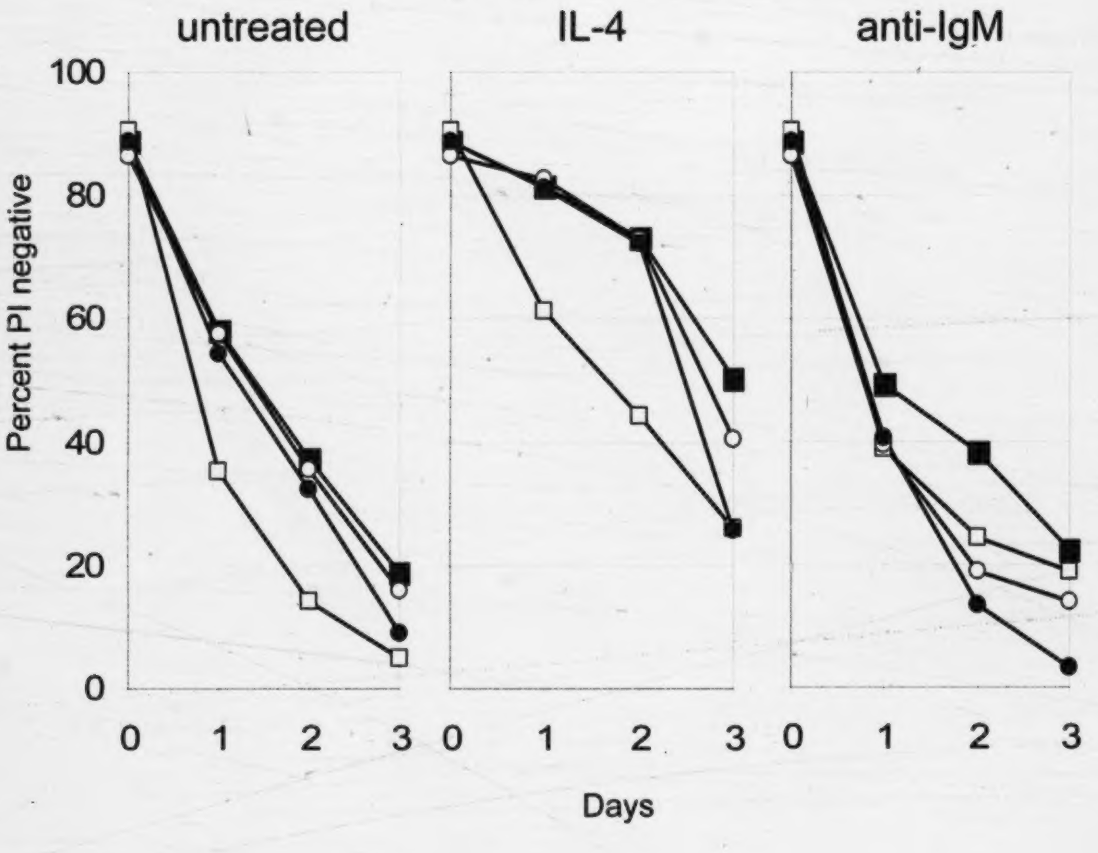

Figure 3-15. ***Rasgrp1*$^{-/-}$; *Rasgrp3*$^{-/-}$ B cells have decreased viability upon BCR ligation.** Splenic B cells were isolated from mice of the indicated genotypes and left untreated or stimulated with either anti-IgM or IL-4. Live cells were quantified by propidium iodide (PI) exclusion 0, 1, 2 or 3 days after the start of the treatment. Genotypes are indicated as in Figure 3-1. Results shown for WT (■), 1KO (□), 3KO (○), and DKO (●) mice.

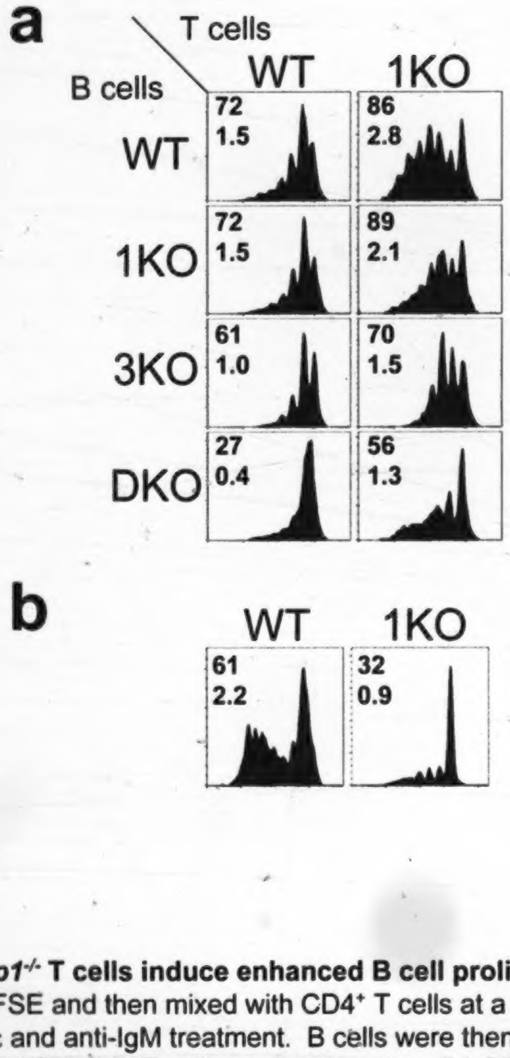

Figure 3-16. *Rasgrp1*$^{-/-}$ T cells induce enhanced B cell proliferation. **(a)** Splenic B cells were labeled with CFSE and then mixed with CD4^{+} T cells at a 1:10, B:T ratio for four days along with anti-CD3ε and anti-IgM treatment. B cells were then analyzed by flow cytometry. B cell genotypes indicated on left, T cell genotypes on top. **(b)** CFSE labeled CD4^{+} T cells were treated with anti-CD3ε for four days. The percentage of cells that divided at least once (top) and average number of cell divisions (bottom) are indicated for each sample. Genotypes are indicated as in Figure 3-1.

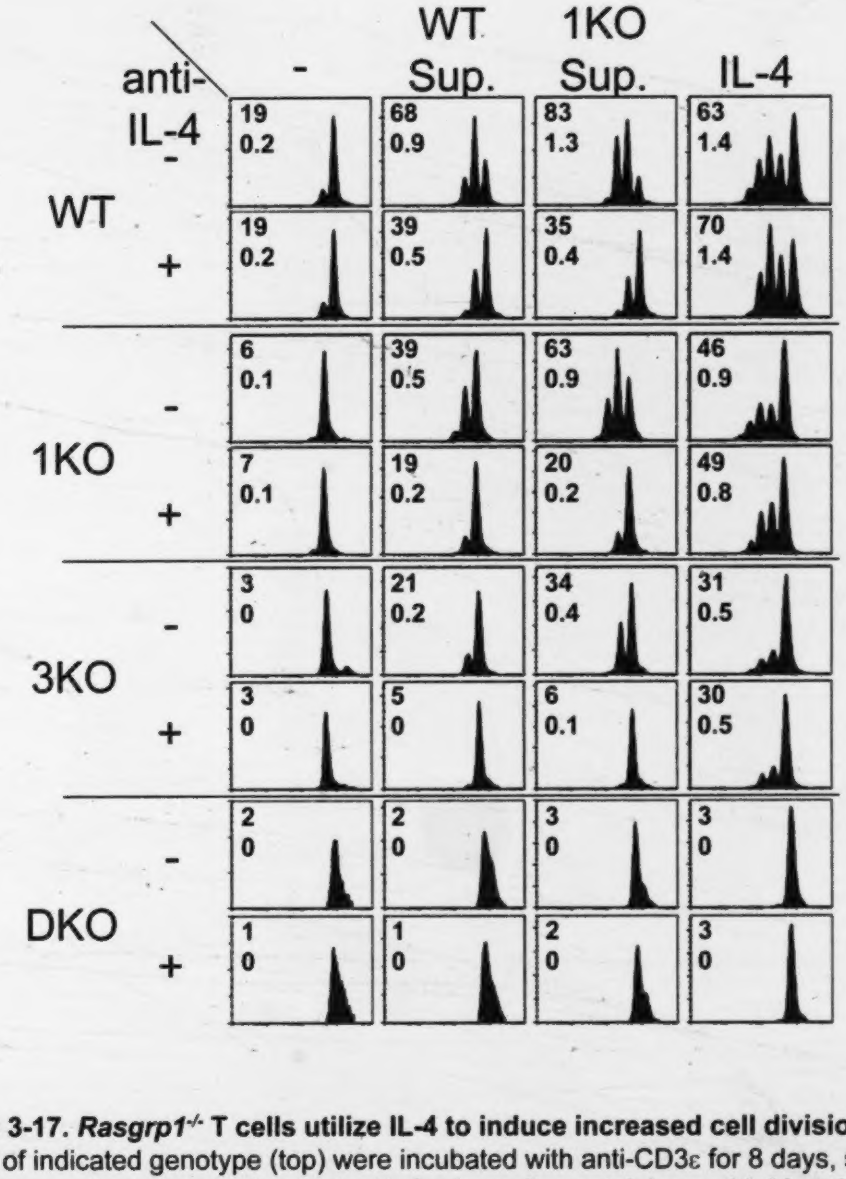

Figure 3-17. *Rasgrp1*$^{-/-}$ T cells utilize IL-4 to induce increased cell division in B cells. T cells of indicated genotype (top) were incubated with anti-CD3ε for 8 days, supernatants (sup.) were collected and mixed 1:1 with fresh media containing anti-IgM and used to stimulate CFSE labeled B cells of indicated genotypes (left). B cells were also treated with anti-IgM alone (-) or with anti-IgM and IL-4 (IL-4). These mixtures were then incubated alone or in combination with anti-IL-4 blocking antibody for four days before being analyzed by flow cytometry. The percentage of cells that divided at least once (top) and average number of cell divisions (bottom) are indicated for each sample. Genotypes are indicated as in Figure 3-1.

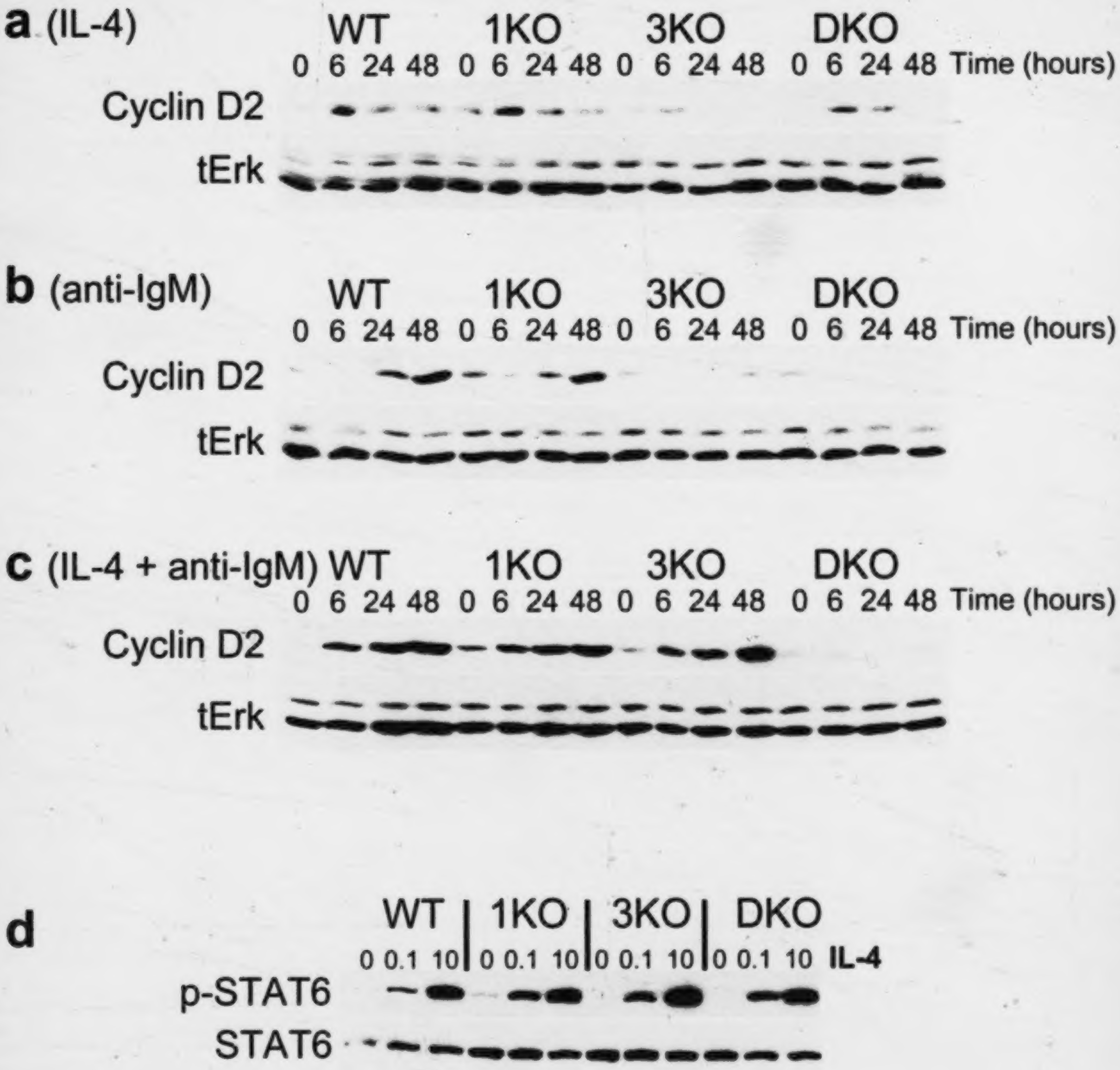
a (IL-4)
b (anti-IgM)
c (IL-4 + anti-IgM)
WT
1KO
3KO
DKO
0 6 24 48 0 6 24 48 0 6 24 48 0 6 24 48 Time (hours)
Cyclin D2
tErk
d
0 0.1 10 0 0.1 10 0 0.1 10 0 0.1 10 IL-4
p-STAT6
STAT6

Figure 3-18. ***Rasgrp1***$^{-/-}$**;** ***Rasgrp3***$^{-/-}$ **B cells fail to upregulate cyclin D2 expression. (a-c)** Splenic B cells were isolated from young mice of the indicated genotypes and treated with **(a)** IL-4 (1 ng/ml), **(b)** anti-IgM (1 ug/ml) or **(c)** a combination of the two for the number of hours indicated. An equal fraction of the starting population was then lysed and subjected to immunoblotting with cyclin D2 and total-Erk (loading) antibodies. **(d)** Splenic B cells were treated with IL-4 (0, 0.1, or 10 ng/ml) and lysates were subjected to immunoblotting with phospho-STAT6 and STAT6 (loading) antibodies. Genotypes are indicated as in Figure 3-1.

3.11 References

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Layer, K., Lin, G., Nencioni, A., Hu, W., Schmucker, A., Antov, A. N., Li, X., Takamatsu, S., Chevassut, T., Dower, N. A., Stang, S. L., Beier, D., Buhlmann, J., Bronson, R. T., Elkon, K. B., Stone, J. C., Van Parijs, L. and Lim, B. (2003). Autoimmunity as the consequence of a spontaneous mutation in Rasgrp1. *Immunity*. **19**(2): 243-255.

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Chapter 4

Discussion

Chapter

4

Discussion

4.1 General discussion

RasGRP1 and RasGRP3 are co-expressed in primary murine B cells. While RasGRP1 is the only RasGRP family member implicated in Ras activation downstream of the TCR, both RasGRP1 and RasGRP3 are responsible for Ras-Erk signaling mediated by BCR ligation in B cells (Figure 4-1). In primary murine splenic B cells, RasGRP3 facilitates the earliest BCR-Ras-Erk signaling, plays the major role in BCR-induced signaling, and is solely responsible for tonic BCR signaling to Ras. B cells express less RasGRP1 than do T cells and within B cells there is less RasGRP1 expressed than RasGRP3, which suggests that RasGRP1 plays a minor role in B cells. In double knockout B cells, PMA-Ras signaling is absent despite significant Erk activation. One possible explanation for this residual signaling to Erk in B cells is PKC-mediated Raf activation, which has previously been documented in other cell types (Kolch et al. 1993; Schonwasser et al. 1998). While this study was in progress, Oh-hora *et al.* generated RasGRP1 and RasGRP3 single and double deficient DT40 chicken B cells (Oh-hora et al. 2003). They determined that in these cells, BCR-Ras signaling utilizes RasGRP3 and not RasGRP1. In addition, these transformed chicken B cells did not display detectable tonic BCR-Ras signaling and, in the absence of both RasGRPs, there was residual Ras-Erk signaling upon BCR-ligation. Thus, the mechanism of BCR-Ras signaling in DT40 cells is somewhat different from that of murine splenic B cells.

4.2 Model of RasGRP1 and RasGRP3 function in the immune system

Mice that lack RasGRP1 expression in their T cells develop a late onset lymphoproliferative and autoimmune syndrome (Layer et al. 2003). While this process complicates the analysis of RasGRP1 and RasGRP3 function in B cells from aged mice, our studies led to the remarkable discovery that the *Rasgrp1*$^{-/-}$ autoimmunity is suppressed in double mutant mice. We have developed a model to conceptualize these findings (Figure 4-2).

4.2.1 Wildtype mice

In wildtype mice, RasGRP1 in T cells mediates Ras activation upon TCR stimulation, allowing for appropriate T cell development and effector function (Figure 4-2). The result is that when these T cells encounter antigen they activate and in turn send co-stimulatory signals to B cells. These T cells also upregulate the expression of Fas-ligand (FasL) upon activation and can kill B cells expressing Fas. In B cells, RasGRP1 and RasGRP3 are expressed allowing for BCR-Ras signaling. This signaling pathway synergizes with RasGRP-independent immune receptor signals and co-stimulatory signals derived from T cell-B cell contact and cytokines. One example of this synergy is at the level of cyclin D2 upregulation in response to stimulation through the BCR and the IL-4 receptor. It has also previously been suggested that Erk signaling in B cells lowers the threshold for other cytokine responses (Chen et al. 1993; Levings et al. 1999). Those B cells that are activated secrete antibodies and send co-stimulatory signals back to T cells, which enhances their proliferative response. Activated B cells that do not receive BCR stimulation are known to be deleted due to the surface expression of Fas, which induces B cell apoptosis when bound to FasL expressed on T cells. Additionally, the production of autoantibodies is further limited in these mice due to the removal of most autoreactive B cells during development. The net effect of these signals is the development and maintenance of a normal adaptive immune system capable of appropriate antibody out-put and pathogen clearance.

4.2.2 *Rasgrp1*$^{-/-}$ mice

In RasGRP1–deficient mice, the absence of TCR-Ras signaling prevents normal T cell development from occurring, resulting in a greatly reduced peripheral T cell population in young mice (Dower et al. 2000). The CD4^{+} T cells that do develop have defective TCR-induced proliferation, Fas-mediated killing of B cells, activation-induced death, and are thought to induce autoimmunity (Figure 4-2) (Layer et al. 2003). While the etiology of the autoimmunity in *Rasgrp1*$^{-/-}$ mice has not been definitively determined (see section 1.6.6), I have shown that the production of anti-nuclear antibodies is thymus-dependent and therefore regulated by T cells. Others have shown that *Rasgrp1*$^{-/-}$ T cells are sufficient to induce splenomegaly (Layer et al. 2003).

Upon TCR stimulation, RasGRP1–deficient CD4^{+} T cells secrete substantially elevated levels of IL-4 *in vitro* (Layer et al. 2003). According to our model, the elevated IL-4 supports the development and survival of autoreactive B cells *in vivo*, thus causing the autoimmunity. Inhibition of TCR-Erk signaling in isolated T cells has been shown to increase IL-4 production in wildtype CD4^{+} T cells by promoting their differentiation into Th2 cells (Dumont et al. 1998; Jorritsma et al. 2003). Similarly, the loss of TCR-Ras-Erk signaling in *Rasgrp1*$^{-/-}$ mice might lead to elevated IL-4 production. Layer *et al.* found that *Rasgrp1* mutant mice have an increased proportion of autoreactive T cells (see section 1.6.6), which might explain the source of the TCR signal that we propose induces IL-4 production *in vivo* (Layer et al. 2003). However, given that IL-4 production is greatly enhanced upon TCR stimulation of isolated CD4^{+} T cells *in vitro*, normal antigen encounter may be sufficient to induce elevated IL-4 secretion *in vivo*.

Several studies of murine autoimmunity have implicated IL-4 signaling in the pathogenic process. Autoimmunity induced by mercuric chloride as well as peptide-induced autoimmunity have both been found to require IL-4 for the maximal generation of anti-nuclear antibodies (Ochel et al. 1991; Deocharan et al. 2003). Administration of anti-IL-4 antibodies into autoimmune-prone NZB/W F1 mice before the onset of disease was found to inhibit the production of anti-dsDNA antibodies, while the addition of IL-4 stimulated splenocytes from littermates enhanced the autoimmune response (Nakajima et al. 1997). As well, constitutive IL-4 expression in transgenic mice is sufficient to induce the production of auto-antibodies (Foote et al. 2004).

Increased production of IL-4 by CD4$^+$ T cells has been reported in LAT^{Y136F} knock-in mice which are unable to activate PLC-γ1 upon TCR stimulation and which also develop autoimmunity (Aguado et al. 2002; Sommers et al. 2002). TCR-Erk signaling is also defective in T cells from lupus patients (Cedeno et al. 2003), consistent with the notion that the T cell defect in *Rasgrp1*$^{-/-}$ mice is the cause of the autoimmunity. Also of note, most of our studies were done on a 129/J x C57BL/6J hybrid background which has been shown to be susceptible to spontaneous autoimmunity (Bygrave et al. 2004). However, I also observed autoimmunity on the C57BL/6J background while crossing *Rasgrp1*$^{-/-}$ mice with Nude mice, although splenomegaly was not consistently present in mice under 6 months of age.

B cells in RasGRP1-deficient mice appear to be largely normal. Despite significantly reduced Ras-Erk signaling upon BCR stimulation, *in vitro* proliferation of these cells is only mildly deficient. Importantly, IL-4 plus anti-IgM-induced proliferation is relatively normal *in vitro*, which we hypothesize allows RasGRP1-deficient B cells to be responsive to the elevated levels of IL-4 produced by the T cells in these mice. Interestingly, the CD138$^+$ population in the spleen *Rasgrp1*$^{-/-}$ mice increases in number prior to the detection of anti-nuclear antibodies. This temporal difference could reflect the time required in order for sufficient autoantibodies to accumulate to the level of detection.

According to our model, *Rasgrp1*$^{-/-}$ CD4$^+$ T cells promote B cell proliferation and autoreactivity by producing abnormally high levels of IL-4. B cells in these mice, which express RasGRP3, have only mildly deficient BCR-Ras signaling *in vitro*. We propose that *in vivo*, BCR signaling is sufficient to allow B cells to respond to antigen plus IL-4 stimulation by upregulating cyclin D2 and proliferating. The activated B cells could then send reciprocal stimulatory signals to the autoreactive $CD4^+$ T cell population, further contributing to the lymphoproliferative condition by allowing both populations to expand. However, given that adoptive transfer of *Rasgrp1*$^{-/-}$ T cells into *Rag*$^{-/-}$ mice is sufficient to induce autoimmunity (Layer et al. 2003), it is quite likely that non-B cells are mediating the expansion of the T cell population. Since *Rasgrp1*$^{-/-}$ $CD4^+$ T cells exhibit decreased capacity for proliferation *in vitro* (Priatel et al. 2002; Layer et al. 2003), I propose that decreased cell death along with homeostatic proliferation both contribute to the accumulation of this cell type *in vivo*. This model partially accounts for the delay in the autoimmunity, as sufficient numbers of IL-4 secreting T cells would be required to accumulate in order to allow autoreactive B cells to survive development and activate.

4.2.3 *Rasgrp3*$^{-/-}$ mice

In *Rasgrp3*$^{-/-}$ mice, T cells are intrinsically normal because they normally lack RasGRP3 expression (Figure 4-2). Since RasGRP1 does not fully compensate for the lack of RasGRP3, B cells from *Rasgrp3*$^{-/-}$ mice have decreased BCR-Ras signaling *in vitro*. BCR plus IL-4 receptor stimulation induces less cyclin D2 expression in *Rasgrp3*$^{-/-}$ B cells than in wildtype cells which correlates with a reduction in proliferation. Unlike wildtype B cells, RasGRP3-deficient B cells do not induce any cyclin D2 production upon stimulation with anti-IgM alone nor do they proliferate at all. According to our model, the wildtype level of co-stimulation provided by $CD4^+$ T cells in these mice is insufficient to rescue this signaling defect. The net result is a modestly decreased proliferative response upon antigen encounter, leading to a moderately diminished serum immunoglobulin levels. T cells in *Rasgrp3*$^{-/-}$ mice might have a subtle defect as an indirect consequence of B cell dysfunction but we have been unable to detect such an effect.

4.2.4 *Rasgrp1; Rasgrp3* double mutant mice

In the double mutant mice, the absence of RasGRP1 leads to defective T cell development and a diminished T cell population in young mice (Figure 4-2). As with *Rasgrp1*$^{-/-}$ mice, these T cells are proposed to be autoimmune and to secrete elevated levels of IL-4. In *ex vivo* double knockout B cells, BCR signaling to Ras was found to be defective, which prevents anti-IgM from synergizing with IL-4 to induce cyclin D2 production. This results in a greatly reduced B cell proliferative response upon BCR ligation *in vitro*, though these cells are still responsive to LPS or anti-CD40 plus IL-4 stimulation. The decreased proliferation of double knockout B cells is proposed to be an important reason for the lack of autoimmunity in these mice as proliferation is known to be required for class-switching and differentiation into antibody secreting cells (Tangye et al. 2004). While double knockout B cells were able to proliferate in response to *Rasgrp1*$^{-/-}$ CD4$^+$ T cells, the level of proliferation was far less than that seen with B cells from RasGRP1-deficient mice.

According to our model, the combined effect of enhanced T cell-derived signals, such as IL-4, along with decreased B cell proliferation, is a diminished B cell effector response as compared to that of *Rasgrp1*$^{-/-}$ mice. We further propose that the decreased sensitivity of B cells to proliferative signals diminishes the production of T cell co-stimulatory signals that would have otherwise come from these B cells. However, given that splenomegaly occurs in *Rag*$^{-/-}$ mice in which *Rasgrp1*$^{-/-}$ T cells have been adoptively transferred it is likely that another cell type is also capable of mediating the T cell co-stimulation that is required for expansion of this population. It remains to be determined if the signal for the expansion of the T cell population arises from B cells or another cell type, though this signal must be deficient in double knockout mice as splenomegaly does not occur. Either way, the result is that the autoreactive CD4$^+$ T cell population remains small in double knockout B cells.

Taken together, the net effect is that the autoimmune and lymphoproliferative disorder seen in *Rasgrp1*$^{-/-}$ mice, and the mild humoral defect seen in *Rasgrp3*$^{-/-}$ mice are balanced in the double mutant mice.

4.3 Isotype specific deficiencies

One significant aspect of the immune response that the model in Figure 4-2 does not address is the immunoglobulin isotype-specific differences observed in the induced immune responses and the aged mice. Of the immunoglobulin isotypes tested, IL-4 is known to induce the production of IgG1, while interferon-γ (IFN-γ) induces IgG2a and IgG3 production (Zhang 2003). However, upon anti-CD3ε stimulation *in vitro, Rasgrp1*$^{-/-}$ T cells produce elevated levels of IL-4 and are defective at IFN-γ production (Layer et al. 2003). This makes it difficult to explain the reduced IgG1 production and normal IgG3 in double knockout mice induced with TD and TI-I antigens, as well as the elevated IgG2a and IgG3 in aged mice. Possibly, other non-T/non-B cell types are involved or the cytokines produced by *in vivo* stimulation differ from those produced *ex vivo.*

A preliminary assessment of cytokines in the serum of aged *Rasgrp1*$^{-/-}$ mice uncovered elevated levels of IFN-γ. Elevated levels of IFN-γ could explain the increased serum IgG2a in aged RasGRP1-deficient mice, although it must be mentioned that the presence of this cytokine has not been confirmed nor has the source been determined.

As with non-immunized mice, the reason for the isotype specific defects seen in the TD and TI-I immunizations are unknown. I have been unable to find another knockout mouse with a similar pattern of isotype specific deficiencies, or a factor that would signal specifically to IgG1 and IgG2a production but not the other immunoglobulin isotypes. PLC-γ2-deficient mice have a similar B cell proliferation defect to *Rasgrp1; Rasgrp3* double knockout mice, however, these mice displayed a different pattern of immunoglobulin deficiency (Wang et al. 2000; Bell et al. 2004). The same can be said for Bam32 and cyclin D2 deficient mice (Solvason et al. 2000; Han et al. 2003). This indicates that a decreased B cell proliferative response is not sufficient to account for the isotype specific deficiencies. It is possible that ablation of the T cell population, or its normalization with a RasGRP1 T cell transgenic, would allow for an analysis of the double knockout humoral immune responses that is easier to interpret.

4.4 B cell development

B cell development appears to proceed normally in the absence of both RasGRP1 and RasGRP3 as judged by surface marker analysis of bone marrow cells and splenocytes. This is in contrast to the severe B cell development defects seen in mice expressing a dominant-negative Ras mutant under the control of a B cell specific promoter (Iritani et al. 1997; Nagaoka et al. 2000). However, as mentioned in section 1.7.3, the developmental defect in Ras^{N17} transgenic mice occurs prior to the expression of the pre-BCR and is likely due to inhibition of IL-7R signaling. Mice deficient in PLC-γ2 have only a mild B cell development defect which further suggests that the effect of Ras^{N17} is on non-BCR mediated signaling (Wang et al. 2000; Bell et al. 2004). Possibly other GEFs, such as Sos, mediate BCR-Ras signaling during development, thus negating the necessity for PLC-γ2, RasGRP1 and RasGRP3. Nonetheless, results from *PLC-γ2*$^{-/-}$ mice suggest that there might be a minor B cell developmental defect in double knockout mice. The *Rasgrp1; Rasgrp3* mutant mice used in this study were on a mixed genetic background, which makes it difficult to observe small effects of *Rasgrp1/Rasgrp3* genotype on development due to the variability of the genetic background of these mice. A more detailed analysis of B cell development can be performed now that the *Rasgrp3*$^{-/-}$ mice have been backcrossed 10 generations to the C57BL/6J genetic background (*Rasgrp1*$^{-/-}$ have already been backcrossed).

The B cell population in the spleen contains predominantly B2 cells (CD5$^-$) while a higher proportion of B1 cells (CD5$^+$) can be found in the peritoneal cavity (Berland et al. 2002). Development of B1 cells is more sensitive to alterations of BCR signaling, which suggests that there might be a diminished population of these cells in double knockout mice. I found a reproducible decrease in both the number and proportion of B1 cells in the spleen that correlated with RasGRP1-deficiency and was present in young and aged mice. The reduced B1 cell population in *Rasgrp1*$^{-/-}$ and double knockout mice could be a result of increased T cell help, which promotes increased B2 cell development (Berland et al. 2002). The number and proportion of splenic B1 cells in *Rasgrp3*$^{-/-}$ mice is similar to wildtype, presumably due to a wildtype level T cell help and the lack of an effect of decreased BCR-Ras signaling on the development of this population.

B1 cells have a more self-reactive BCR repertoire than B2 cells (Berland et al. 2002). The decreased B1 cell population in *Rasgrp1*$^{-/-}$ mice rules out any contribution of this population to the autoimmunity in these mice. The development of B1 cells in the peritoneal cavity of *Rasgrp1, Rasgrp3* single and double mutant mice has not been studied. This portion of B cell development should also be assessed on the C57BL/6J background.

4.5 Role of tonic Ras activation in B cell survival and anergy

RasGRP3 is required for maintaining the basal level of Ras activation in untreated cells. This low level of Ras-Erk signaling has been shown to be mediated by B-Raf but not Raf-1 (Brummer et al. 2002) and may arise due to tonic signaling from the BCR. Tonic signaling, which is mediated by BCR surface expression and shares many of the hallmarks of antigen-induced BCR signaling, is required for survival of mature B cells (Kraus et al. 2004; Monroe 2004). Kraus *et al.* eliminated BCR expression in mature B cells, using Cre expression under the control of the CD21 promoter and *loxP*-flanking the genes responsible for Igα and immunoglobulin heavy chain expression. Upon B cell maturation, BCR expression is lost due to expression of Cre which causes the deletion of BCR components by genomic recombination. Mature B cells from these mice are greatly decreased in number due to reduced survival. However, it is unlikely that Ras activation upon tonic signaling serves in B cell survival *in vivo* given that the survival of untreated double knockout and *Rasgrp3*$^{-/-}$ B cells in our *in vitro* studies is similar to wildtype.

Stimulation with anti-IgM induces increased death of *Rasgrp3*$^{-/-}$ and double knockout B cells as compared to wildtype although this is not the case if the cells are left unstimulated. Immediately following isolation, the naïve B cells recovered from mice of all four genotypes have equivalently high levels of viability (normal scatter properties and low propidium iodide staining), roughly equivalent IgM expression, and do not contain activated (CD69$^+$) or plasma B cells (CD138$^+$). Therefore, the decreased viability of mutant B cells cannot be accounted for by an altered composition of B cells in the starting population.

B cells from Ras^{N17} transgenic mice display reduced survival *in vivo*, although this death is independent of BCR-stimulation and likely a result of reduced IL-7-mediated signaling (Nagaoka et al. 2000). PLC-γ2 is also required for survival of unstimulated B cells (Bell et al. 2004). The selective survival deficiency of double knockout B cells in response to anti-IgM suggest the presence of pro-apoptotic BCR-mediated signals in the absence of anti-apoptotic signals that would normally be mediated by the RasGRPs. Ras is known to activate several anti-apoptotic signaling cascades (Cox et al. 2003) and BCR-signaling is known to induce pro-apoptotic signals (Graves et al. 2004).

Isolated *Rasgrp1*$^{-/-}$ B cells display reduced viability relative to wildtype, which is independent of stimulation. Unlike anti-IgM treated double knockout B cells which have an increased rate of death, after the first day of culture the death rate of RasGRP1-deficient B cells is similar to that of wildtype B cells regardless of treatment. Given the reduced viability of *Rasgrp1*$^{-/-}$ as compared to double knockout B cells, the autoimmunity and the increased death are likely related. The method I used to assess cell death detects only late stage apoptosis, which means that the *Rasgrp1*$^{-/-}$ B cells that die during the first day of culture could already be undergoing the early stages of apoptosis. Assessment of an earlier apoptotic marker would allow this point to be clarified.

Given the low number of T cells in young mice, survival of *Rasgrp1*$^{-/-}$ B cells is not likely dependent upon T cell contact. Similarly, the fact that IL-4 treatment does not rescue these cells would suggest that these cells do not die due to cytokine removal. However, one must note that in these treatments IL-4 is not added until after the B cells are isolated, at which point some of the cells could have already begun an irreversible process towards apoptosis. The addition of IL-4 during the isolation procedure would allow the contributions of this cytokine to *Rasgrp1*$^{-/-}$ B cell viability to be assessed.

Anergic T cells lack TCR-Ras signaling and do not proliferate upon TCR stimulation (see section 1.6.5). Anergic B cells also have decreased proliferative responses but unlike anergic T cells they have increased basal Erk phosphorylation and weak BCR-Erk signaling (Healy et al. 1997; Rui et al. 2003). These experiments used transgenic mice that express both a hen egg white lysozyme (HEL)-specific BCR and soluble HEL resulting in a decreased splenic B cell population of which the cells remaining are anergized (Goodnow et al. 1988). Upon the addition of HEL, splenic B cells isolated from these transgenic mice have diminished antibody secretion induced by non-BCR signals. The inhibitory effect of adding HEL to these anergic cells is abrogated by the addition of a Mek inhibitor, indicating that Erk activation is required for transmission of BCR-mediated anergic signaling. Anergic B cells and RasGRP1; RasGRP3 double knockout B cells are both insensitive to BCR-mediated proliferation but respond to anti-CD40 plus IL-4 stimulation (Cooke et al. 1994). However, double knockout B cells do not have elevated tonic Erk activation, suggesting that they are not truly anergic. Despite the dissimilarities between these two cell types, other forms of B cell anergy may possibly result in a phenotype similar to that of double knockout B cells. Another interesting point is that RasGRP1 expression can be downregulated in chronically PMA-stimulated T cells, while RasGRP3 expression is maintained in chronically PMA-stimulated B cells. This RasGRP expression pattern in T cells and B cells correlates with the status of the Ras pathway in each of the respective anergic cell types.

PKCδ-deficient mice develop autoimmunity (Mecklenbrauker et al. 2002; Miyamoto et al. 2002). While negative selection of B cells is normal in these mice, peripheral tolerance is not established (Mecklenbrauker et al. 2002). PKCδ has been shown to phosphorylate RasGRP3 and phosphorylation of this protein is required for maximal activation (Aiba et al. 2004; Brodie et al. 2004; Zheng et al. 2005). Therefore, PKCδ-meditated RasGRP3 phosphorylation may be an important component in the establishment and maintenance of B cell anergy and could explain the elevated level of phosphorylated Erk in anergic cells. However, *Rasgrp3*$^{-/-}$ mice do not develop autoimmunity suggesting that either PKCδ phosphorylation of RasGRP3 is not required for the development of anergy or that the decreased responsiveness of *Rasgrp3*$^{-/-}$ B cells negates the need for the development of B cell tolerance.

4.6 Cyclin D2 induction and proliferation of B cells

BCR ligation, with or without IL-4 co-stimulation, results in cyclin D2 induction and a weak induction of cyclin D3 expression (Solvason et al. 1996; Lam et al. 2000; Solvason et al. 2000; Glassford et al. 2001; Piatelli et al. 2004). Double knockout B cells fail to upregulate the expression of cyclin D2 upon anti-IgM plus IL-4 treatment, which very likely prevents a normal proliferative response from occurring. Interestingly, B cells from cyclin D2 deficient mice do not proliferate in response to BCR stimulation and yet have relatively normal anti-CD40- and LPS-induced proliferative responses (Solvason et al. 2000). This means that anti-CD40 and LPS can induce non-cyclin D2 pathways for proliferation, while anti-IgM treatment cannot. LPS has been shown to induce robust cyclin D2 and cyclin D3 expression (Lam et al. 2000) while in one study anti-CD40-mediated signaling was found to induce cyclin D2 but no cyclin D3 expression (Lam et al. 2000). However, the anti-CD40 antibody used by Lam and colleagues failed to induce a substantial B cell proliferative response in either wildtype or cyclin D2-deficient mice. The antibody used by Lam *et al.* was not the one I used in my studies and the lack of a proliferative response induced by their antibody is unlike what is observed when CD40-ligand is used to stimulate B cells (Solvason et al. 2000; Hasbold et al. 2004). Stimulation of transformed B cells using CD40-ligand was found to induce the expression of both cyclin D2 and cyclin D3, with IL-4 greatly enhancing the induction of cyclin D3 (Grdisa 2003). It is likely that physiological stimulation of B cells through CD40 results in activation of both cyclin D2 and cyclin D3. Thus, anti-IgM induces cyclin D2 expression in order to induce proliferation, while the anti-CD40 antibody that I used and LPS both induce cyclin D2 plus cyclin D3 expression. Taken together the results argue that RasGRP1 and RasGRP3 are required for sustained cyclin D2 induction by anti-IgM +/- IL-4.

BCR-induced cyclin D2 expression is blocked by inhibitors of PI3K, Mek, and NF-κB (Piatelli et al. 2002; Piatelli et al. 2004), indicating that multiple signaling pathways are involved in cyclin D2 regulation. The promoter of cyclin D2 contains an AP-1 binding site which explains the role of Ras-Erk signaling in the induction of cyclin

D2 expression (Brooks et al. 1996). However, there are no STAT6 binding sites in the cyclin D2 promoter. Thus, either STAT6 binds to a non-consensus site or one of the many transcription factors whose expression is induced by STAT6 is responsible for the transcription of this gene. Schroder *et al.* used a GeneChip to identify STAT6-regulated genes (Schroder et al. 2002). They found seven transcription factors whose expression is decreased and five that are increased by the loss of STAT6 expression. Therefore, STAT6 could bind directly to the cyclin D2 promoter, induce the expression of a transcription factor that activates the promoter or decrease the expression of a repressor that inhibits the promoter. Interestingly, the cyclin D3 promoter also contains AP-1 binding sites (Brooks et al. 1996), and yet RasGRP-deficiency does not affect LPS-induced proliferation.

In double knockout B cells, there is a 50 percent reduction in the proliferative response induced by anti-CD40 treatment. The RasGRP contribution to CD40 effector function could be mediated by tonic BCR signaling to the cyclin D2 promoter synergizing with CD40 signaling or RasGRPs could be directly involved in a CD40-mediated signaling. As mentioned in section 1.7.4, there is substantial evidence for a CD40-PLC-RasGRP-Erk pathway, although there also appear to be other mechanisms for CD40-Erk signaling. Possibly, the alternative BCR-Erk pathway induced by prolonged CD40 treatment (Mizuno et al. 2005) results in enhanced tonic BCR-Erk signaling that synergizes with CD40 signaling. Since double knockout B cells have reduced tonic BCR-Ras-Erk signaling, the CD40-mediated enhance BCR tonic signaling could be insufficient to synergize with other direct CD40 signaling pathways. Indeed, there is enhanced Erk phosphorylation in cells treated with CD40 ligand for 48 hours (Mizuno et al. 2005). Whether this elevated level of Erk activity is mediated directly by CD40 ligand stimulation or by means of enhanced tonic signaling has not been established. However, Mizuno *et al.* demonstrated that the enhanced basal Erk activation is blocked by PI3K inhibitors, while these inhibitors only have a minor effect on CD40-mediated Erk activation and the enhanced BCR-Erk signaling induced by pretreatment with CD40 ligand. Therefore, the anti-CD40-mediated proliferation deficiency in double knockout mice may be due to RasGRP1 and RasGRP3 functioning in tonic BCR signaling, CD40-

mediated signaling, or both. Further studies will be required to elucidate the role of the RasGRPs in CD40-mediated effector functions.

4.7 RasGRP3-mediated Rap signaling

PMA-induced Rap1 activation was unaffected in *Rasgrp3*$^{-/-}$ and double knockout mice, while SDF-1-induced B cell migration was only mildly deficient. There is conflicting evidence for RasGRP2 expression in B cells (Crittenden et al. 2004; Roose et al. 2005). Thus, there may be redundancy in the activation of Rap in B cells mediated by RasGRP2 and RasGRP3. Indeed, RasGRP2 overexpression in DT40 cells potentiated BCR-Rap1 signaling (Gold et al. 2000). The contribution of RasGRP2 towards Rap activation in B cells could be assessed using mice deficient in this protein which have already been generated by Dr. Ann Graybiel's group (Crittenden et al. 2004). They found that RasGRP2 is required for maximal PMA- and thrombin-induced Rap activation in platelets as well as for integrin-dependent aggregation. Given the decreased PMA-Rap signaling in these mice, RasGRP2 likely contributes to Rap activation in B cells. However, there is not a complete ablation of PMA-induced Rap activation in *Rasgrp2*$^{-/-}$ platelets, suggesting that RasGRP3 may also play a role. The analysis of Rap activation in B cells should be performed with *Rasgrp2; Rasgrp3* single and double mutant mice. In addition, other Rap GEFs could contribute to Rap activation in B cells. Initial studies suggested that C3G might mediate Rap activation upon BCR stimulation by means of C3G-Crk complexes binding to tyrosine phosphorylated Cbl and Cas (Ingham et al. 1996). However, further studies discounted this possibility since the formation of these complexes could be observed in the absence of Rap activation and vice versa (McLeod et al. 1998).

While PMA-induced Rap1 activation was normal in *Rasgrp3*$^{-/-}$ B cells, we do not know if this mutation has an effect on BCR-Rap signaling. PMA-induced Ras activation is only mildly deficient in *Rasgrp3*$^{-/-}$ B cells while BCR-Ras signaling is significantly affected. This differing phenotype at the level of Ras activation suggests a similar phenotype might be seen with Rap activation and that anti-IgM induced Rap activation should be assessed. Interestingly, BCR- and PMA-induced Rap activation is required for integrin-mediated adhesion and actin polymerization in B cells (McLeod et al. 2004). Any deficiency in these Rap-mediated processes would decrease B cell activation and B cell: T cell interactions. Therefore, a decrease in BCR-Rap signaling in double knockout B cells could be a contributing factor for the suppression of autoimmunity.

4.8 Future directions

There are numerous questions remaining regarding the role of RasGRP1 and RasGRP3 in the immune system. For instance, we do not know which Ras effectors other than Raf are activated by RasGRP1- or RasGRP3-dependent mechanisms. Nor have we identified the hypothetical B cell to T cell co-stimulatory signal that allows for T cell expansion in *Rasgrp1*$^{-/-}$ mice. *Rasgrp1* and *Rasgrp3* mutant mice will be of great value in answering many other questions regarding the role of the RasGRPs in the immune system.

4.8.1 Role of RasGRPs in autoimmunity

The deficiency of RasGRP1 in T cells has not been definitively established to be sufficient to induce autoimmunity nor has the additional loss of RasGRP1 plus RasGRP3 in B cells been proven sufficient to prevent this autoimmunity. Other cell types that normally express RasGRP1 and or RasGRP3 might also be involved in autoimmunity and its suppression. The cell type-specific contribution of the RasGRPs to the generation and suppression of autoimmunity could be assessed by adoptive transfer of T cells plus B cells from wildtype and mutant donors into *Rag*$^{-/-}$ mice. These experiments require that all mice utilized have the same genetic background and are now possible since *Rasgrp3*$^{-/-}$ mice on the C57Bl/6J genetic background are now available.

Cell transfer experiments could also be used to assess the contribution of homeostatic proliferation to the development of autoimmunity in *Rasgrp1*$^{-/-}$ mice. King *et al.* found that lymphopenia-induced homeostatic proliferation leads autoimmunity in NOD mice (King et al. 2004). They inhibited homeostatic proliferation by injecting NOD mice with sufficient numbers of T cells isolated from NOD littermates. This injection inhibited the onset of diabetes in these mice. Similarly, the contribution of homeostatic proliferation to the onset of autoimmunity in *Rasgrp1*$^{-/-}$ mice could be assessed by the injection of additional T cells isolated from mice of the same genotype.

While double knockout mice do not develop autoimmunity, we do not know whether the mutation of both *Rasgrp1* and *Rasgrp3* in B cells alone is sufficient to suppress other forms of autoimmunity. In order to test this, RasGRP1-deficiency in T cells could be rescued by the T cell-specific transgenic expression of RasGRP1 (*Rasgrp1*^{Tg}) which has already been developed by others (Norment et al. 2003). Provided autoimmunity does not occur in *Rasgrp1*$^{-/-}$*; Rasgrp1*^{Tg} mice, these mice could be mated to *Rasgrp3*$^{-/-}$ mice along with various autoimmune mice to determine the impact on the generation of autoimmunity.

4.8.2 Role of RasGRP1 and RasGRP3 in other cell types

Both the BCR and the TCR use ITAM motifs to signal through a kinase cascade to PLC, which then activates a RasGRP family member. There are other immune receptors that also contain ITAM motifs and signal to PLC plus Ras, raising the possibility that a RasGRP family member is activated downstream of these receptors. To date, RasGRP1 and RasGRP3 signaling has only been analyzed in a limited number of cells. This is due not only to the limited expression data in primary cells, but also to the lack of rapid recognition of the possible role of the RasGRPs in signaling systems previously attributed to the PKC family (Brose et al. 2002). The potential for RasGRPs to be involved in PKC-dependent signaling pathways is due not only to members of both protein families having DAG and calcium binding domains, but also to the discovery that PKC-mediated phosphorylation is required for maximal RasGRP3 activation (Teixeira et al. 2003; Aiba et al. 2004; Zheng et al. 2005). In order to determine in which signaling pathways the RasGRPs play a role, the expression of these proteins in various cell types must first be determined.

Expression analysis could be accomplished by fluorescently labeling RasGRP antibodies that have been developed in our lab and using them as staining antibodies in flow cytometry. This technique would allow for the analysis of RasGRP1 plus RasGRP3 expression in cell populations that would be extremely difficult to purify in sufficient quantities for western blotting. This technique would also allow one to observe changes in relative protein levels during development and differentiation.

Based on preliminary experiments looking at RasGRP expression in primary cells and cell lines, there are several candidate pathways in which RasGRPs are likely to play a role. For instance, others in the lab have found that Natural Killer (NK) cell lines express RasGRP1 but not RasGRP3. Murine NK cells express DAP12, a 12 kDa protein that contains an ITAM motif and associates with NK cell activating receptors (NKARs) (Campbell et al. 1999). Ligation of Ly49D (an NKAR) results in PLC-γ1 and Erk activation (McVicar et al. 1998) which suggests a role for RasGRP1 in this signaling pathway. The Fc-gamma receptor I (FcγRI) pathway in macrophages is likely to utilize a RasGRP family member to mediate Ras activation. FcγRI complexes contain ITAMs (Ravetch et al. 2001) and ligation of these complexes results in PLC, PKC and Erk activation (Melendez et al. 1999; Myung et al. 2000). Therefore, RasGRP1 and/or RasGRP3 may be required for macrophage signaling as well. In addition to macrophages, many others cells express FcγRI receptors which may provide a role for the RasGRPs in a number of other cell types (Ravetch et al. 2001).

4.9 Conclusion

This dissertation provides evidence that the RasGRPs are important regulators of the immune system that link antigen recognition to proliferation and differentiation. Upon BCR ligation, RasGRP1 and RasGRP3 mediate Ras activation. This signaling allows for the coupling of antigen receptor signaling with cyclin D2 induction, which induces proliferation of B cells. Proliferation is important for the generation of a proper immune response, as it allows for the expansion and differentiation of the pathogen-specific B cell population. The analysis of mice deficient in RasGRP1 and RasGRP3 led to the remarkable discovery that the *Rasgrp3* null mutation could suppress autoimmunity in *Rasgrp1*$^{-/-}$ mice. *Rasgrp1*$^{-/-}$ T cells promote greater B cell proliferation by secreting elevated levels of IL-4. *Rasgrp3*$^{-/-}$ B cells lack BCR ligation-induced proliferation due to the absence of cyclin D2 induction. In double knockout mice, the elevated T cells cytokine secretion and reduced B cell proliferation are balanced such that these mice have a relatively normal immune response.

This study raises the possibility that inhibition of RasGRP1 and RasGRP3 could allow for the modulation of the immune system in patients with autoimmune disorders. While it has not been determined if the mildly defective hapten-specific immune response in double knockout mice is equivalent to an immune response that is sufficient to clear pathogens, the restricted expression of the RasGRPs and the relatively normalized immune response in double knockout mice, suggests that these proteins are ideal targets for therapeutic inhibition.

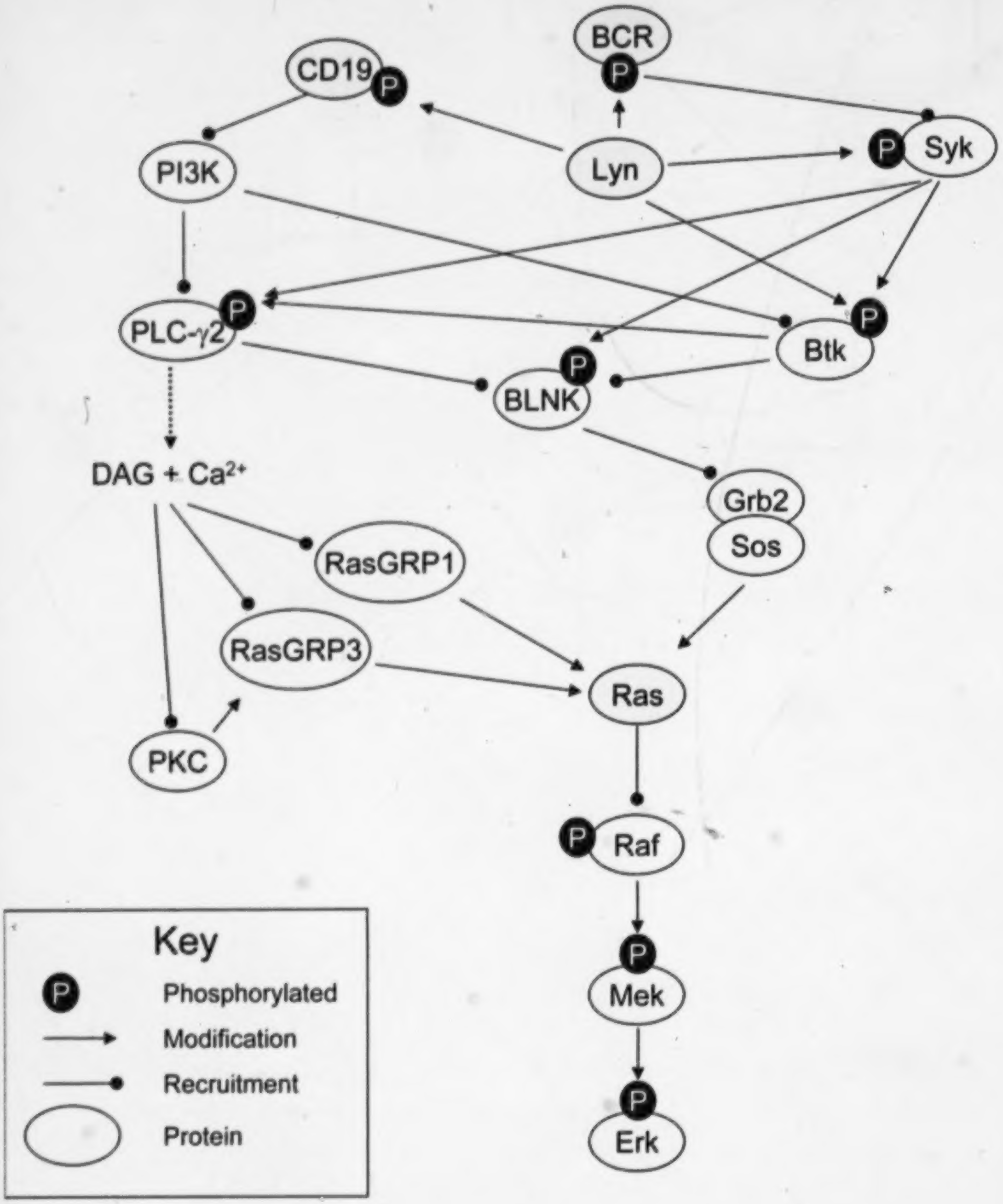
BCR
P
CD19
P
PI3K
Lyn
P
Syk
PLC-γ2
P
P
Btk
P
BLNK
DAG + Ca2+
Grb2
Sos
RasGRP1
RasGRP3
Ras
PKC
P
Raf
P
Mek
P
Erk
Key
P
Phosphorylated
Modification
Recruitment
Protein

Figure 4-1. RasGRP1 and RasGRP3 link BCR to Ras. For simplicity, signaling which does not directly lead to Ras activation as well as divergent signaling at the level of Ras have been omitted. Grey background of proteins denotes constitutive membrane association. See key for other representations used and text for details.

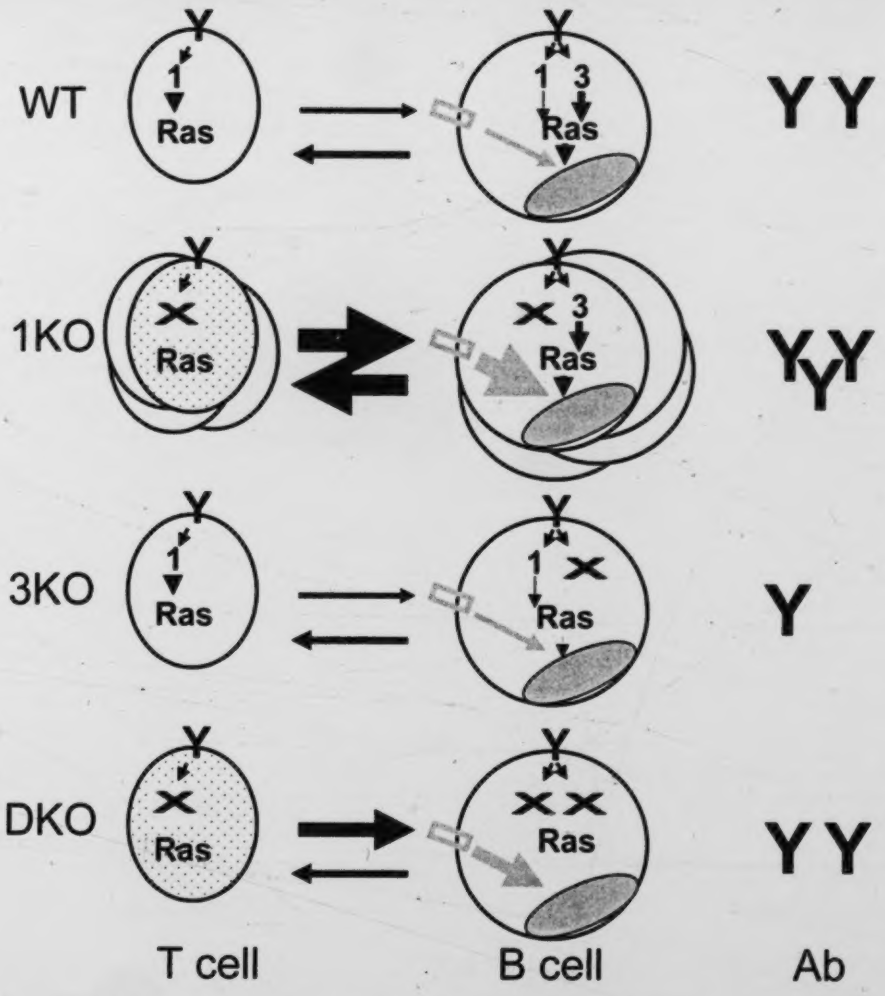

Figure 4-2. Model for RasGRP1 and RasGRP3 function in autoimmunity.
In wildtype T cells (WT), Ras is activated by RasGRP1 (1) downstream of the TCR (Y). Communication between T cells and B cells (horizontal arrows) sends activation signals to each cell (omitted in T cells; in B cells signaling through receptors, grey open box, leads to signaling, grey arrow). Downstream of the BCR (Y), RasGRP3 (3) plays a greater role than RasGRP1 in Ras activation. This signal synergizes with signals from T cells at the level of nuclear events (grey zone), resulting in B cell proliferation and antibody secretion (large Y). In *Rasgrp1*$^{-/-}$ mice (1KO) abnormal T cell development, caused by RasGRP1 deficiency (X), generates autoreactive T cells which send greater co-stimulatory signals (thicker arrow) causing increased B cell activation. These B cells express RasGRP3 and are thus able to respond to this stimulus with increased proliferation and enhanced T cells co-stimulatory signals (thicker arrow). In *Rasgrp3*$^{-/-}$ mice (3KO) T cells are normal, while B cells are less responsive to stimuli due to decreased Ras activation downstream of the BCR resulting in decreased B cell proliferation and antibody production. In double knockout mice (DKO), autoreactive T cells send increased stimulatory signals to B cells that are less responsive due to the loss of Ras activation downstream of the BCR. The net result is antibody production similar to that seen in wildtype mice, and limited T cell co-stimulation arrising from B cells and other cell types (omitted). This model was generated in collaboration with Dr. James C. Stone.

4.11 References

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